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The Haem–Globin Linkage

1. ARTIFICIAL HAEMOGLOBINS FROM HAEMATINS LACKING FREE CARBOXYL GROUPS

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(*Received* 20 *May* 1959)

Differing opinions have been expressed as to which groups of the haems are essential for coupling to globin. Thus Haurowitz & Waelsch (1929) claimed to have prepared from native globin and dimethylmesohaemin (mesohaemin with esterified propionate groups) a haemoglobin capable of reversible combination with molecular oxygen, and Haurowitz (1949) described use of the same compound to demonstrate that ferrohaemoglobin contained a water molecule co-ordinated with the iron. On the other hand, Granick & Gilder (1946) considered that 'the co-ordination of the central iron atom is not sufficiently strong by itself to bind the haem', and Blyumenfeldt (1952) believed that the main linkages were between carboxyl groups of the haems and basic groups in the globin.

In an attempt to reconcile the conflicting concepts: (1) that an iron–protein link was adequate, and (2) that haem propionate–protein links were necessary, Lemberg & Legge (1949) suggested that the ester might have been hydrolysed in the process of combination. To settle in an unequivocal manner whether haematins lacking free carboxyl groups could combine with native globin, aetiohaemin (with inert methyl and ethyl side chains, see Fig. 1) was employed, some preliminary results with which have already been reported (O'Hagan, 1950, 1951, 1955). Haurowitz's finding that artificial haemoglobins can be prepared from methylated haemins has been confirmed and the studies have been extended to experiments which substantiate the hypothesis that whereas the essential linkage for oxygenation is through the iron atom, linkages through the haem propionate side chains may be necessary to impart stability.

METHODS AND MATERIALS

Hydrogen-ion concentration. This was measured with a Jones electronic pH meter (N. L. Jones, Melbourne) calibrated with the phthalate, phosphate and borate buffers described in British Standard 1647 (1950).

Paper chromatography. The technique of Nicholas & Rimington (1949) was used chiefly to ensure that no pigments with free carboxyl groups contaminated the specimens of aetio and methylated porphyrins and haemins. That the spots ran with the solvent front (R_F 1) was interpreted as meaning that no free carboxyl groups were present, but, of course, did not, without reference to other evidence, prove that the specimens were essentially pure compounds. For the coproporphyrin preparation, the chromatography of the esters as described by Chu, Green & Chu (1951) was performed as a check on purity as the extraction proceeded. The technique was performed at 21° and it was found best to view the spots under u.v. light before the solvent dried.

Measurement of spectra. A Hilger Uvispek spectrophotometer was employed; the wavelength scales were calibrated against a mercury arc, the extinction scales against the copper sulphate and cobalt sulphate solutions of Gibson (1950). The glass prism was used for all experiments reported here; the wavelength error over the range 390–1100 mμ was ±1Å and, after realignment of the attenuator network, the extinction scales were within the manufacturer's tolerance of ±0·0016 unit.

A Beck Hartridge reversion spectroscope was checked for wavelength accuracy with a mercury-arc lamp; the scale was true to ±1Å over the range 450–650 mμ.

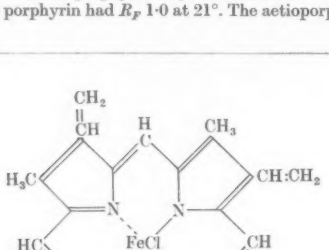
Chemicals. Methanol A.R. grade was used for the combination experiments. Sodium dithionite (British Drug Houses Ltd.) was dispensed, on opening a 500 g. bottle, into a number of ½ oz. MacCartney bottles. A fresh one of these bottles was used as soon as the granules showed any tendency to stick together, which was taken as an indication of deterioration.

Aetiohaemin III. The method of Schumm (1928, 1929) was used to prepare several batches of the aetioporphyrin from protohaemin, with a yield of 4·6%. The heating in liquid paraffin B.P. (1958) was performed in 250 ml. batches in a 500 ml. round-bottom Pyrex flask fitted with a short wide air condenser, heating with a Tower's Isomantle being controlled by a Simmerstat to keep the temperature between 344 and 352° for 80 min. The flask was occasionally shaken during the period. The porphyrin was twice recrystallized from $CHCl_3$–methanol and gave the following absorption bands in solution (Hartridge reversion spectroscope). In 10% HCl: I, 590·5; II, 547·4 mμ (order of intensity: II > I). In $CHCl_3$: I, 620·0; II, 570·1; III, 532·7; IV, 497·6 mμ (decreasing order of intensity: IV, III, II, I). In ether: I, 622·3; II, 611·8; III, 597·8; IV, 577·9; V, 569·8; VI, 559·0; VII, 527·3; VIII, 496·2 mμ (decreasing order of intensity: VIII, VII, V, I, IV, III, II, VI). Bands III, II and VI were of very low intensity and were just detectable in saturated solutions. They probably originate from a trace of another porphyrin as impurity. In conc. H_2SO_4: I, 592; II, 549 mμ (order of intensity: II > I). These measurements compared well with those given by Schumm (1929) and by Fischer & Treibs (1930).

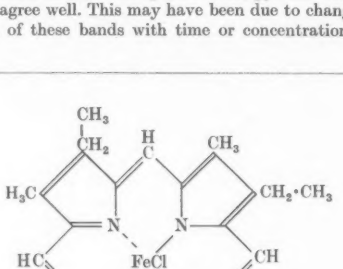
On paper chromatography with lutidine–water–NH_3 by the method of Nicholas & Rimington (1949) the porphyrin repeatedly gave a single spot with R_F 1·0 at 21°. Kehl & Stich (1952), using 2:4-lutidine–water at 15°, found R_F 0 for aetioporphyrin (isomer unspecified), with slight movement from the point of application at 25°, but they found that copro- and uro-porphyrin esters moved with R_F 1·0. The difference could be due to the isomer used, or result from adsorption on application to the paper. It may perhaps be explained by the finding of R_F 0 when $CHCl_3$ was used as a solvent for the aetioporphyrin, for when pyridine was employed the porphyrin ran with the solvent front; the presence or absence of an NH_3 atmosphere made no difference with this porphyrin. By the method of Chu *et al.* (1951) the porphyrin had R_F 1·0 at 21°. The aetioporphyrin was completely insoluble in dilute aqueous alkali, further indicating no trace of pigments with carboxylic acid groups.

The haemin was prepared from the porphyrin by introducing iron by the method of Zalcski (1904). The absorption spectra then obtained were as follows. In $CHCl_3$: I, 641·0; II, 543·0; III, 501 mμ onward (decreasing order of intensity: II, I, III). In pyridine: I, 632·0; II, 556·5; III, 521·5 mμ measured immediately (decreasing order of intensity: III, II, I). In pyridine + hydrazine hydrate: I, 546·3; II, 515·3 mμ (order of intensity: II > I).

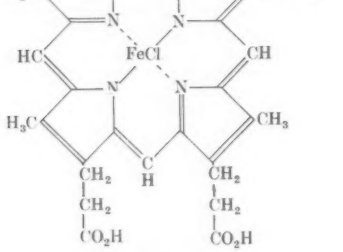
The spectrum in $CHCl_3$ was close to that given by Fischer & Orth (1937) but the spectra of the pyridine solutions did not agree well. This may have been due to change of position of these bands with time or concentration or both.



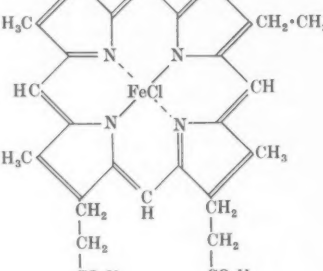
Protohaemin IX



Mesohaemin IX



Aetiohaemin III



Coprohaemin III

Fig. 1. Structures of haemins mentioned in text.

Chromatography with lutidine–water–NH_3 gave R_F 1·0 at 21°, and no traces of haemins soluble in aqueous alkali were present.

Dimethylprotohaemin IX. Two methods of preparation were employed: A, esterification of haemin with methanolic HCl by the method of Küster & Schlayer (1927); B, by insertion of iron into the dimethylprotoporphyrin prepared by the method of Grinstein (1947). The porphyrin gave absorption maxima agreeing with those of Grinstein, namely: I, 631·1; II, 575·0; III, 541·3; IV, 507·4; V, 407·3 mμ (decreasing order of intensity: V, IV, III, I, II). Lutidine–water–NH_3 chromatography gave a single spot, R_F 1·0 at 21°. Iron was introduced by solution of the porphyrin in boiling $CHCl_3$ and addition of a freshly prepared boiling ferrous acetate solution in acetic acid saturated with NaCl. After cooling, the dimethylprotohaemin was taken into ether, the acid removed with dilute sodium acetate solution and then water, and the ether was evaporated.

Both preparations gave the following absorption spectra. In $CHCl_3$: I, 635·8; II, 547·5; III, 505 mμ (decreasing order of intensity: II, I, III). In pyridine + hydrazine hydrate: I, 556·8; II, 527·0 mμ onward (order of intensity: II > I). The second spectrum compared fairly well with that given by Fischer & Orth (1937), namely: I, 560; II, 527·5 mμ, but the spectrum in $CHCl_3$ (as with that obtained above for actiohaemin in pyridine) was considerably different from that listed by Fischer & Orth, namely: I, 625; II, 565; III, 515 mμ. Both preparations A and B gave the same spectral peaks, so it is most likely that the Fischer & Orth figures are in error. Chromatography of preparation A at 21° with lutidine–water–NH_3 gave one major component with R_F 1·0 and a trace of a component with R_F 0·7, probably some free protohaemin. Preparation A was used for the combination experiments with globin.

Dimethylmesohaemin IX. Mesoporphyrin IX was prepared from dimethylprotoporphyrin IX by the method of Grinstein & Watson (1943). It had, on chromatography with lutidine–water–NH_3, R_F 0·8 at 21°. It was converted into the dimethyl ester by treatment with methanolic HCl overnight, the mixture diluted with water, the ester taken into $CHCl_3$, the solution dried with anhydrous Na_2SO_4 and crystallized after addition of methanol and concentration on the hot plate. The ester gave a single spot with R_F 1·0 on chromatography with lutidine–water–NH_3 at 21°. Addition of iron, as described for dimethylprotohaemin, did not alter the R_F of the single spot obtained, and it was completely insoluble in dilute aqueous alkali.

Tetramethylcoprohaemin III. The porphyrin was prepared from the $Mg(OH)_2$ adsorbate of the porphyrins obtained during the preparation of *Corynebacterium diphtheriae* toxoid as described by Gray & Holt (1948). The adsorbate was supplied through the courtesy of Mr R. W. Patterson of the Commonwealth Serum Laboratories, Melbourne. During the extraction it was found that some of the red pigment present was not behaving as a normal porphyrin and could not be removed with acid, but was soluble in 2% (w/v) NaOH. After passage through ether and removal of all fluorescent porphyrin with 2–10% (w/v) HCl, the pigment could be eluted from the ether with 2% NaOH and precipitated from this by acidification with HCl. In the 2% NaOH the spectrum had peaks at 564·0 and 529·2 mμ. After being washed and dried, the precipitated pigment dissolved in dioxan had peaks at 562·3 and 526·3 mμ, which suggested that the pigment was essentially copper coproporphyrin, since a solution of this complex porphyrin prepared by the method of Fischer & Orth (1937) from some of the free coproporphyrin from the main batch had peaks in the same position. Enquiry from Mr Patterson revealed that the method used by Gray & Holt had been modified to increase the toxoid yield by the addition of 10 ml. of 1% (w/v) $CuSO_4$/l. of medium, as recommended by Mueller (1939). The free porphyrin could be obtained from the copper complex by solution in conc. H_2SO_4, immediate dilution with water and precipitation at pH 3·4. The porphyrin was esterified with methanolic HCl, chromatographed on $CaCO_3$ from benzene and extracted with $CHCl_3$–benzene (1:10, v/v). This chromatographed material from the copper-complex fraction still contained a white impurity of waxy nature and was not used for later work. Paper chromatography of the porphyrin by the method of Chu, *et al.* (1951) revealed two spots, a major one with R_F 0·63 and a minor one with R_F 0·37 (probably from tetramethylcoproporphyrin and hexamethylcarboxylic acid porphyrin). Spots with these values and one of 0·21 (octamethyluroporphyrin I) were obtained from the crude porphyrin of the main batch before chromatography on $CaCO_3$.

The main batch of the porphyrin ester (the first from the $CaCO_3$ column) was rechromatographed on $CaCO_3$ and recrystallized from methanol–$CHCl_3$. After drying in a vacuum desiccator over silica gel, the solid had m.p. 147°, with remelt at 179·5° (uncorr.), which compared well with the values of Jope & O'Brien (1945).

To prepare the haemin, 10 mg. of the ester was dissolved in acetic acid saturated with NaCl, and freshly prepared ferrous acetate in acetic acid was added at the boiling point. No crystals appeared after 2 days, so $CHCl_3$ was added, the mixture washed with water and the $CHCl_3$ evaporated. The tetramethylcoprohaemin III was then used without further purification. It was completely insoluble in aqueous alkali.

Coprohaemin III. A portion (5 mg.) of tetramethylcoprohaemin III was heated with 10% (w/v) NaOH for several hours (Fischer & Orth, 1937), the hydrolysate of the ester precipitated with dilute HCl and the free coprohaemin taken into pyridine. Crystallization was attempted from acetic acid saturated with NaCl. After 2 weeks, with gradual evaporation of the acetic acid, no crystals had appeared, so ether and water were added; the coprohaemin precipitated in the aqueous phase and was collected, dried over NaOH under vacuum and used without further purification.

The peaks in the absorption spectra from the free coprohaemin were: in pyridine: I, 557; II, 524·5 mμ (order of intensity II > I); in pyridine + hydrazine hydrate: I, 547; II, 518 mμ onward (order of intensity II > I). These compare well with the values of Schumm (1927) for the pyridine haemochrome.

Native globin. Considerable difficulty was experienced with this preparation since the methanol used to effect attachment of the haematins, the sodium dithionite used for reduction and changes in H^+ ion concentration often precipitated traces of denatured globin that were present in solution, and made exact spectrophotometric studies impracticable. The addition of buffers in the presence of methanol, although permissible with apomyoglobin (O'Hagan & George, 1960), produced clouding, so buffered

solutions were not used, the ionic strength being kept as low as possible and the pH measured after haematin combination had occurred. Several methods of globin preparation were tried, that of Jope, Jope & O'Brien (1949) proving most suitable. However, after adjustment of the native globin solution to the isoelectric point of the denatured material (pH 7·8), addition of $(NH_4)_2SO_4$ to 50% saturation and precipitation of denatured globin, a perfectly clear solution was obtained, but, after dialysis at 1° to remove $(NH_4)_2SO_4$ [in the equipment described by O'Hagan (1959*a*), adjustment to the isoelectric point at room temperature (21°) brought down more precipitate]. Rossi-Fanelli, Antonini & Caputo (1958*a*), preparing globin from crystalline human haemoglobin, also found this precipitation when the solutions were brought to room temperature. The concentration of the globin was measured by the micro-Kjeldahl technique. All the work reported here was performed with human globin. The reputedly more stable horse and ox globin were tried but gave similar trouble.

EXPERIMENTAL AND RESULTS

Aetiohaemoglobin, mesohaemoglobin and derivatives

For some experiments, a solution of 5 mg. of aetiohaemin in 3·5 ml. of warm methanol was first made, chilled to 0° and added slowly to 50 ml. of 0·34% globin in water in a 100 ml. conical flask that was swirled in ice–water. The solution was allowed to come to room temperature, filtered and spun at 18 000 *g* for 10 min. to remove uncombined aetiohaemin. Since a variable fraction of the aetiohaemin precipitated, and since from the absorption curves it appeared that another fraction did not combine (but did not precipitate), it was considered unjustifiable to attempt to obtain quantitative absorption curves or determine extinction coefficients. Therefore most curves were obtained by adding a saturated solution of the haemin in methanol to a slight excess of native globin solution and centrifuging. The best absorption curves obtained for the oxyaetiohaemoglobin and the ferroaetiohaemoglobin are shown in Fig. 2.

The spectra closely resembled those obtained from mesohaemoglobin derivatives in the position of the absorption peaks but were not so sharp, as can be seen from Fig. 3.

Difficulty was experienced with the dithionite, as has been noted by Dalziel & O'Brien (1957), who found that even native haemoglobin was irreversibly affected. Numerous attempts failed to obtain the α- and β-oxyhaemoglobin peaks at the same relative height as in normal oxyhaemoglobin. This may have been due to the presence of some uncombined aetiohaemin or of a compound of the type described by Dalziel & O'Brien. Recombination appeared best at about pH 5·5–6·0, but this may have been because at lower H^+ ion concentrations traces of denatured protein precipitated.

The oxyaetiohaemoglobin appeared more unstable than oxymesohaemoglobin and auto-oxidized more rapidly. That a true oxyhaemoglobin was present was confirmed by the reversion of the two-banded spectrum to the one-banded reduced spectrum when the solution was placed under a vacuum of 5 mm. Hg for 45 min., and the reappearance of the oxyhaemoglobin type of spectrum on admission of air.

Carboxyaetiohaemoglobin

By reduction of a solution of ferriaetiohaemoglobin with dithionite and the passage of coal gas for a few minutes carboxyhaemoglobin was obtained with absorption curve as shown in Fig. 4.

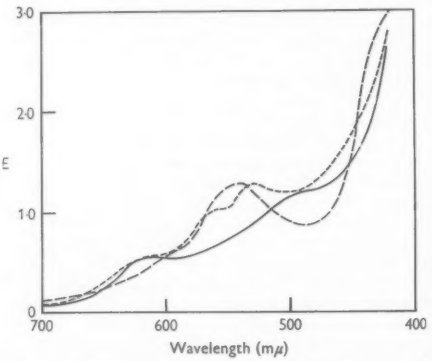


Fig. 2. Qualitative absorption spectra of aetiohaemoglobin derivatives: ferriaetiohaemoglobin (——), ferroaetiohaemoglobin (– – –), oxyaetiohaemoglobin (- - - -). pH 5·6; no added buffer.

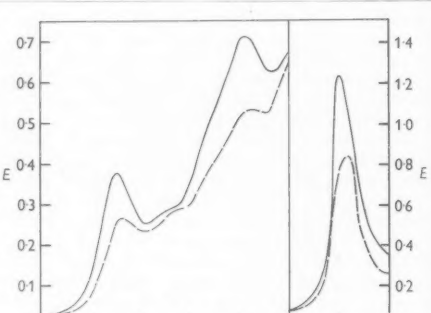


Fig. 3. Qualitative absorption spectra of ferrimesohaemoglobin (——) and ferriaetiohaemoglobin (– – –). pH 7·0, no added buffer; solution was clarified by centrifuging at 12 000 *g* for 5 min.

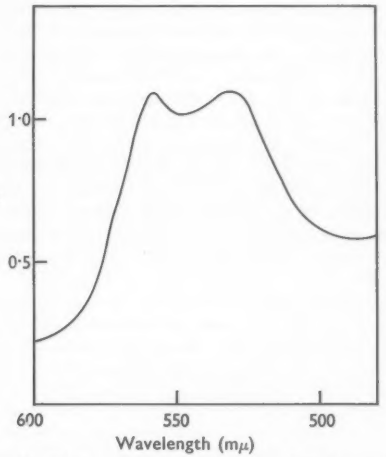


Fig. 4. Qualitative absorption spectrum of carboxyaetiohaemoglobin. pH 7·0, no added buffer.

Dimethylprotohaemoglobin and derivatives

Dimethylprotohaemoglobin and the corresponding dimethylmesohaemoglobin were prepared by dissolving the haemins in methanol to form saturated solutions and adding 5 ml. of such solutions to 50 ml. of 0·34% globin solution, under the same conditions as for aetiohaemoglobin. The position of the peaks found for the dimethylmesohaemoglobin were the same as those of Haurowitz & Waelsch (1929), and for dimethylprotohaemoglobin they were as follows:

ferridimethylprotohaemoglobin, 631·5 mμ;
ferrodimethylprotohaemoglobin, 561·5 mμ;
oxydimethylprotohaemoglobin, I, 576·8,
II, 541·0 mμ (Hartridge reversion spectroscope).

Oxytetramethylcoprohaemoglobin III and oxycoprohaemoglobin III

Combination experiments with tetramethylcoprohaemin III and coprohaemin III gave similar spectra to those obtained with the dimethylmesohaemin, as follows:

oxycoprohaemoglobin III, I, 568; II, 533·5 mμ;
oxytetramethylcoprohaemoglobin III, I, 567;
II, 532 mμ (Hartridge reversion spectroscope).

Reduction with sodium dithionite gave the typical broad-banded spectra of the ferrohaemoglobins. Unfortunately precipitation set in on the several occasions when these experiments were carried out, so that it was not possible to obtain a reliable absorption curve, and the small quantity of the haemins available did not permit further attempts.

DISCUSSION

The question whether it is legitimate to draw conclusions from experiments with recombined and artificial haemoglobins, in order to interpret the structure of native haemoglobin, must be considered. There would appear to be abundant evidence that recombination can produce a haemoglobin very closely resembling the native molecule. Roche & Combette (1937) observed resemblance in crystalline form, oxygen affinity, solubility in ammonium sulphate solutions and molecular weight (by osmotic-pressure measurements). Gralén (1939) found the same molecular weight (diffusion measurements), pH-stability diagram and molecular shape (ultracentrifuge sedimentation). However, slight differences showed up on electrophoresis (Gralén, 1939), on splitting with bases (Roche & Combette, 1937) and on exact ultraviolet spectroscopy (Hill & Holden, 1926; Jope, 1949). Jope *et al.* (1949) prepared a crystalline recombined haemoglobin spectroscopically identical with native haemoglobin, and it is very likely that the other differences would have been resolved had their method of preparation been used by earlier workers. In fact, Rossi-Fanelli, Antonini & Caputo (1958*b*) have claimed to have prepared such recombined haemoglobin, identical with native haemoglobin in absorption spectra, isoelectric point, electrophoretic mobility, sedimentation and diffusion, stability to heat and alkali, haem–haem interaction, Bohr effect and oxygen affinity.

It would therefore appear reasonable to interpret experiments with other haematins in place of protohaematin on the basis that corresponding haemoglobins are formed in those cases where similar spectral-absorption curves are obtained. It should be pointed out, however, that no experiments have yet been reported to show that haemoglobins prepared from these haematins have the same molecular weight as protohaemoglobin, and, since globin (mol.wt. 34 000; Gralén, 1939) dimerizes on recombination with protohaematin, it may well be that some of the side chains effect this change.

Oxyhaemoglobins can be prepared from mesohaemin and haematohaemin (Hill & Holden, 1926), rhodohaemin, diacetyldeuterohaemin, *Spirographis* haemin and pheohaemin b (Warburg & Negelein, 1932), so that it is evident that vinyl groups are not essential for reversible oxygenation, at least *in vitro*. Callaghan & O'Hagan (1949) noticed differences in the rate of reduction and subsequent uptake of oxygen when recombined ferriproto- and ferrimeso-haemoglobin were incubated with ascorbic acid at pH 7·2 in 0·05M-phosphate buffer. The ferriprotohaemoglobin took 15 min. and the meso-compound 90 min. to change to the oxyhaemoglobin. Rossi-Fanelli *et al.* (1958*a*) found

evidence that the vinyl groups influenced haem–haem interaction, and Rossi-Fanelli & Antonini (1957), working with myoglobin, discovered that deuteromyoglobin had a much higher oxygen affinity than recombined protomyoglobin.

Clearly, the haematin side chains have a marked effect on the oxygenation reaction. One of the problems associated with the formation of the oxyaetiohaemoglobin described above has been the need to use about three times as much dithionite to reduce the aetiohaemoglobin as had been required with the proto- and meso-haemoglobin. This probably represents differences in the oxidation–reduction potential of the iron atom and suggests that the vinyl and propionate groups may interact to provide the ideal supplement to the resonating structure of the porphin ring to allow physiological oxygenation to occur. Oxyaetiohaemoglobin at pH 5·6 required 45 min. at 5 mm. Hg to remove most of the oxygen (O'Hagan, 1959*b*), whereas recombined oxyprotohaemoglobin lost its oxygen in less than 1 min. at the same pressure and hydrogen-ion concentration.

Granick & Gilder (1946) considered that the vinyl groups played a role in the insertion of iron into the porphyrin nucleus, since they found that whereas protoporphyrin, protohaemin, mesohaemin, haematohaemin and deuterohaemin supported the growth of *Haemophilis influenzae*, mesoporphyrin, haematoporphyrin and deuteroporphyrin did not. Since to insert the iron two hydrogens on the pyrrole nitrogens have to be displaced, the vinyl groups may exert influences along the resonating structures in the same way as they affect the oxygen affinity of the haem iron in the haemoglobins and myoglobins. The finding of Granick & Gilder (1946) that esterified haemins and dimethylprotoporphyrin did not support growth may be due simply to their insolubility in the media.

There have been many suggestions that the haem lies in a cleft in the globin molecule. This was implied by Conant (1932–33) and advanced by St George & Pauling (1951), but the ability to form an active oxyhaemoglobin from tetramethylcoprohaemin III, although not conclusive evidence, does appear to support the argument of Keilin (1953) that the haems lie on the surface and not in a cleft or trough into which only haems of the dimensions of protohaem can fit. Of course, another explanation would be that there is a certain amount of flexibility of the polypeptide chains, which may have been displaced sufficiently to allow the larger molecule to enter. In any case, this means that if haemoglobin biosynthesis proceeds from porphyrins or haematins with longer side chains than those of protohaematin, attachment of these precursors to the globin or to its precursor could occur (O'Hagan, 1955).

George & Lyster (1958) have discussed the evidence for a crevice-positioning of the haems in haemoglobin. They consider that the concept of a structure comprising one bond from the protein to the iron is the most satisfactory, with the uncertainty whether a part of the protein shields the other side of the haem from reaction with large ligands. Recently, fresh evidence has suggested (O'Hagan, 1959*c*) that the residue in the protein responsible for the linkage to the haem iron may be a carboxyl group rather than an imidazole nitrogen, as proposed by Conant(1932–33) and widely accepted.

That active haemoglobins can be formed from aetiohaemin and methylated haemins, although indicating that the essential linkage is through the iron atom, does not mean that the propionate groups are unnecessary for haemoglobin function. Rather, the instability of oxyaetiohaemoglobin suggests that these groups may have an influence in consolidating the molecule and the importance of haem propionate–protein linkage in ferrihaem-albumin (O'Hagan, 1955, 1959*b*) indicates the possibility of a similar type of linkage in haemoglobin and myoglobin. Blyumenfeldt (1952) considered them of major importance in effecting haem linkage to globin, based on his work on the 'transhaematination reaction', in which the haematin is transferred from haemoglobin to gelatin, on incubation. Hill & Holden (1926), Holden (1937, 1941), Granick (1949) and Lemberg & Legge (1949) consider such linkages possible. The existence of bonds of this type is also implied by George & Hanania (1954).

The poor spectral extinction of the aetiohaemoglobin compared with the mesohaemoglobin derivatives is no doubt related to the similar differences described by Haurowitz (1927), for the corresponding pyridine haemochromes. In chloroform, in which solvent interaction with the propionate groups is unlikely, aetiohaemin and mesohaemin have essentially the same extinction in the visible and Soret region (J. E. O'Hagan, unpublished work).

It is probable that, with mesohaematin, combination of the propionate groups with either basic groups in the protein or pyridine occurs, enhancing the extinction. The extinction of native haemoglobin derivatives correspond much more closely to those of the artificial haemoglobins prepared from mesohaematin than to those from aetiohaematin. This strongly suggests that the normal linkage in haemoglobin is through haem propionate groups in addition to those through the iron atoms.

SUMMARY

1. Artificial haemoglobins capable of reversible oxygenation can be prepared from aetiohaemin III, dimethylprotohaemin IX, tetramethylcopro-

haemin III and coprohaemin III. The work of Haurowitz & Waelsch (1929), who prepared an active haemoglobin from dimethylmesohaemin IX, has been confirmed.

2. The indications are that the linkage essential for oxygenation is through the iron atom but the possibility of linkages through the haem propionate groups, imparting further stability to the linkage and to the whole protein structure, is suggested by the instability and poor spectral extinction of the artificial haemoglobins prepared from haemins lacking free carboxyl groups.

3. That tetramethylcoprohaemin III can give rise to an active haemoglobin lends support to the suggestion that the haems are on the 'surface', and that linkage to the globin of haematin precursors of larger dimension than protohaematin could occur during haemoglobin biosynthesis.

I am most grateful to Dr A. E. Shaw and the Blood Transfusion Committee of the Queensland Division of the Australian Red Cross Society for providing facilities and giving encouragement to further this work. Professor R. Lemberg generously gave advice on the preparation of the haemins. Mrs Teresa Hamilton and Miss Cecily Barnett gave their willing technical assistance.

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The Haem–Globin Linkage

2. ARTIFICIAL MYOGLOBINS FROM HAEMATINS LACKING FREE CARBOXYL GROUPS

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In the preceding paper (O'Hagan, 1960) it was shown that artificial haemoglobins could be prepared from aetiohaemin III, dimethylprotohaemin IX, dimethylmesohaemin IX, tetramethylcoprohaemin III and coprohaemin III. These studies have been extended for the first three pigments to the preparation of artificial myoglobins.

MATERIALS AND METHODS

Haemins. The preparations of aetiohaemin III, dimethylprotohaemin IX and dimethylmesohaemin IX were the same as used for the preceding paper.

Horse ferrimyoglobin. The method of Theorell (1932) as modified by Lawrie (1951) was used. Three precipitations with $(NH_4)_2SO_4$ were performed, the solutions dialysed free of sulphate and stored in polythene bottles at $-10°$ with 2% NaCl added, as suggested by Hanania (1953).

Apomyoglobin. This was prepared by the method of Theorell & Åkeson (1955). Into the cups of a refrigerated centrifuge at $-15°$ were placed tubes each containing 40 ml. of acetone–HCl (2 ml. of M-HCl/l. of acetone) at the same temperature. A portion (2 ml.) of 1% (w/v) ferrimyoglobin solution was injected into each tube, the tubes were stoppered and shaken, allowed to stand for a few minutes until the protein had coagulated and then were spun at 4000 *g* for 5 min. The supernatant haemin solution was then decanted, the tubes were inverted on filter paper to drain for 5 min., and then 2 ml. of ice-cold water was added to each and the apomyoglobin dissolved. The apomyoglobin solution from eight tubes was pooled, placed in a cellulose dialysis tube ($1\frac{1}{2}$ in. diam.) and dialysed in the cold at 1° against dilute $NaHCO_3$ solution (50 mg./l.), the equipment described by O'Hagan (1959*b*) being used. A small amount of denatured protein was precipitated, and was removed by high-speed centrifuging (16 000 *g* for 10 min.); by contrast, globin solutions showed heavy precipitation at this stage. The apomyoglobin solutions were stored at 1° and could be kept for 3 weeks. The concentration of protein was determined by u.v. spectrophotometry at 280 mμ, with the E_{mM} of 13 obtained by Hanania (1953). There was some indication that this figure may have been too low (P. George & J. E. O'Hagan, unpublished work) but this would not have affected the present work since the protein was used in excess of the haemins. A small trace (not more than 1·5%) of unsplit myoglobin was present. Use of more acid for splitting or longer contact with the acetone–HCl removed more haematin but more denaturation of the protein occurred.

Recombination of haematin with apomyoglobin. To ensure that all haematin capable of combination did so, the apomyoglobin was used in excess. In a typical experiment, 0·3 ml. of freshly prepared 1·5 mM-haemin solution (9·78 mg. of protohaemin + 1 ml. of 0·1 N-NaOH to dissolve, with 9 ml. of water added) was introduced with rapid mixing into 10 ml. of 0·15 mM-apomyoglobin solution in phosphate buffer (pH 7·0, *I* 0·05). The mixture was set aside for 1 hr. before reading.

Fluoride derivatives. These were prepared by adding NaF in considerable excess. To 2 ml. of the ferrimyoglobin solutions was added 1 ml. of a filtered saturated solution of NaF (~1 M).

Spectrophotometry. A Beckman model DU spectrophotometer with photomultiplier attachment was employed, the extinction scale was checked against the copper sulphate and cobalt sulphate standards of Gibson (1950) and the wavelength scale against a mercury arc.

Hydrogen-ion concentration measurements. A Beckman model G pH meter, standardized against the phthalate, phosphate and borate buffers described in British Standard 1647 (1950), was employed.

RESULTS

Recombined ferrimyoglobin. The Soret peaks of native and recombined ferrimyoglobin are shown in Fig. 1, and indicate that about 96% of the haematin had recombined when added in 0·01 N-NaOH to excess of apomyoglobin in phosphate buffer (pH 7·0, *I* 0·05).

The clearest indication of recombination to an active haemoglobin was demonstrated by conversion into the fluoride complex. This avoids the use of dithionite, which partly denatures the protein. Fluoride does not combine with haematin or ferrihaemalbumin [where linkage of haematin is through the carboxyl groups (O'Hagan, 1955, 1959*b*)]. The curves obtained are shown in Fig. 2, again indicating about 96% recombination.

Combination of aetiohaemin with apomyoglobin. In this case combination was achieved by adding the aetiohaemin in methanol to the apomyoglobin solution in phosphate buffer at 0°.

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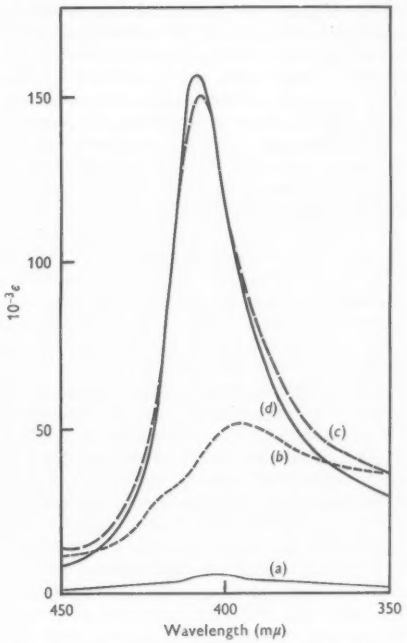

Fig. 1. Absorption spectra showing effect of addition of protohaematin to apomyoglobin in phosphate buffer, pH 7·0, I 0·05. (*a*) Apomyoglobin, (*b*) haematin in 0·01 N-NaOH; (*c*) after addition of haematin to excess of apomyoglobin; (*d*) original ferrimyoglobin. Recombination was as described in the text.

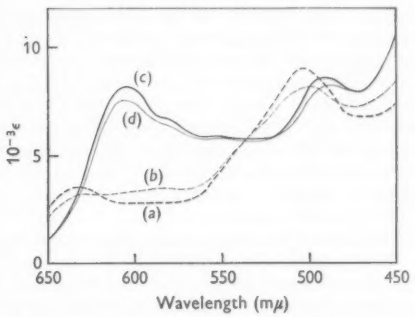

Fig. 2. Absorption spectra of myoglobins in phosphate buffer, pH 7·0, I 0·05. (*a*) Native ferrimyoglobin; (*b*) recombined ferrimyoglobin; (*c*) native ferrimyoglobin fluoride; (*d*) recombined ferrimyoglobin fluoride. Recombination was as described in the text. Fluoride was added to 0·3 M concentration.

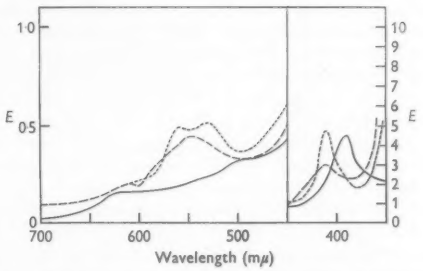

Fig. 3. Absorption spectra of actiomyoglobin derivatives in phosphate buffer, pH 7·0, I 0·05. Ferri-, ——; ferro-, — — —; carboxy-, – – – –; 3 ml. of 68 μM-apomyoglobin + 0·116 mg. of actiohaemin in 0·1 ml. of methanol. Dilution, 1:10 with buffer for Soret-region measurements; 1 cm. cell.

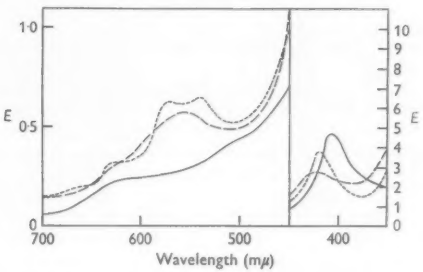

Fig. 4. Absorption spectra of dimethylprotomyoglobin derivatives in phosphate buffer, pH 7·0, I 0·05. Ferri-, ——; ferro-, — — —; carboxy-, – – – –; 3 ml. of 68 μM-apomyoglobin + 0·134 mg. of dimethylprotohaemin in 0·1 ml. of methanol. Dilution, 1:10 with buffer for Soret-region measurements; 1 cm. cell.

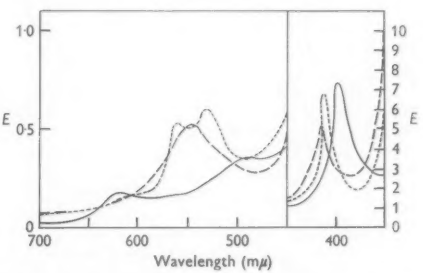

Fig. 5. Absorption spectra of dimethylmesomyoglobin derivatives in phosphate buffer, pH 7·0, I 0·05. Ferri-, ——; ferro-, — — —; carboxy-, – – – –; 3 ml. of 68 μM-apomyoglobin + 0·134 mg. of dimethylmesohaemin in 0·1 ml. of methanol. Dilution, 1:10 with buffer for Soret-region measurements; 1 cm. cell.

The ferri-, ferro- and carboxy-compounds had absorption curves with peaks corresponding to those described by Theorell & Åkeson (1955) for protomyoglobin, but were displaced as would be expected. The spectra for the artificial myoglobin derivatives are shown in Fig. 3. The aetiohaemin and apomyoglobin were 68 μM in the final solution, the solution being diluted 1:10 with the phosphate buffer for the Soret-band measurements.

Combination of dimethylprotohaemin and dimethylmesohaemin. Artificial myoglobins from these pigments were obtained in a similar fashion at the same concentration as for aetiohaemin. The curves are shown in Figs. 4 and 5.

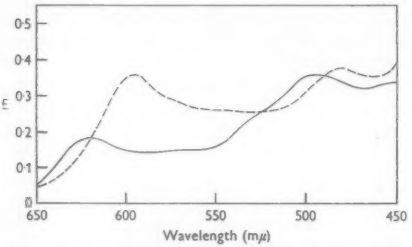

Fig. 6. Absorption spectra of dimethylmesomyoglobin derivatives in phosphate buffer, pH 6·2, *I* 0·05. Ferri-, ——; fluoride, — — —. Concn., ~60 μM-dimethylmesomyoglobin; fluoride was added to 0·3 M concn.; 1 cm. cell.

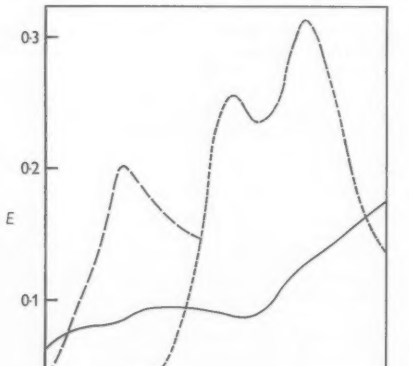

Fig. 7. Absorption spectra of dimethylmesomyoglobin derivatives in phosphate buffer, pH 8·0, *I* 0·05. Ferri-, ——; fluoride, — — —; carboxy-, – – –. Concn., ~30 μM-dimethylmesomyoglobin; fluoride was added to 0·3 M concn., 1 cm. cell.

The solutions of the ferrimyoglobins of Figs. 3–5 were set aside for 4 hr. at 22° before measurement and conversion into the other derivatives.

Fluoride compounds of ferridimethylmesomyoglobin. Since the curves from dimethylmesomyoglobin appeared sharper than those from the others, this compound was used for further studies, the combination with fluoride at pH 6·2 and 8·0. Figs. 6 and 7 clearly show that combination had taken place in both instances.

DISCUSSION

The tendency for apomyoglobin to be more stable than globin and to be more readily cleared of denatured material allowed the use of phosphate buffer solutions, made these studies much easier than those reported in the preceding paper (O'Hagan, 1960) and allowed investigation of the fluoride complexes.

Rosenfeld & Surgenor (1950) found that haematin in 0·01 N-sodium hydroxide lost the ability to form ferrihaemalbumin to the extent of 7% in 1 hr. This probably explains why about 4% of the haematin used here did not combine with the apomyoglobin, as can be seen from Figs. 1 and 2.

With aetiohaemoglobin derivatives (O'Hagan, 1960) it was noted that the absorption curves had peaks that were less sharp than those from the corresponding haemoglobins (proto- and meso-) with free carboxyl groups. A similar lessened intensity of extinction was observed here with the aetio-, dimethylproto- and dimethylmeso-myoglobin derivatives. This might suggest the absence of a linkage which in the normal haemoproteins enhances the extinction.

The slightly sharper peaks for the derivatives of dimethylmesohaemin might suggest that more complete combination had occurred. The small hump at about 630 mμ in the carboxydimethylprotomyoglobin suggests that there was some free haematin present, as does the flatness of the curve for the ferric derivative. That the ester had not been hydrolysed during the combination experiments was shown for dimethylmesomyoglobin by splitting off the haematin with acetone–hydrochloric acid. The haematin was found to be insoluble in alkali, indicating that the carboxyl groups were still esterified.

The fluoride compounds are most interesting since free haematins do not give fluoride complexes under similar experimental conditions. This specific reactivity of the compounds from substituted haematins provides convincing proof, like the oxygenation of the corresponding artificial ferrohaemoglobins, that combination has occurred between the haematin and the protein. Furthermore, it is strong evidence for the formation of an iron–protein bond. These fluoride reactions are also

significant because, in similar experiments with artificial ferrihaemoglobins, denatured protein was precipitated, making it impossible to judge whether a reaction had occurred.

Rossi-Fanelli & Antonini (1957) have shown that deuterohaemin can also combine with apomyoglobin to give an active oxymyoglobin, but the oxygen affinity was much higher, though the Bohr effect was similar to that of the native myoglobin. Clearly, as with haemoglobin, variation of the side chains influences the reactivity towards oxygen and it would appear that protohaem represents the most suitable prosthetic group for the physiological activity of haemoglobin and myoglobin.

SUMMARY

1. Artificial myoglobins have been prepared from aetiohaemin III, dimethylprotohaemin IX and dimethylmesohaemin IX.

2. The spectra for the ferric, ferro and carbon monoxide complexes of these myoglobins are given.

3. Fluoride complexes of ferridimethylmesomyoglobin can be formed at pH 6·2 and 8·0, providing further evidence that combination through the haematin iron had taken place.

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Fatty Acids of Human Blood

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Peters & Man (1943) studied the fatty acid composition of human blood. Brun (1939) and Kirk (1938) observed that esterified and free cholesterol in blood cell was different from that in plasma in healthy individuals. Studies by Chevallier, Manuel & Rouillard (1951) and Evans, Waldron, Oleksyshyn & Riemenschneider (1956) have indicated that the partition of the unsaturated fatty acids in blood cells was quite different from that in plasma. Alkali-isomerization spectrophotometric methods (Hammond & Lundberg, 1955; Luddy, Barford & Riemenschneider, 1958) have been employed to determine the polyunsaturated fatty acids of human blood plasma, the component lipids of human plasma and atheroma. Very few workers have, however, analysed the component fatty acids of human blood cells, plasma and whole blood. This paper describes an investigation into the component lipids and fatty acids of whole blood, plasma and blood cells of healthy men, alkali-isomerization spectrophotometric methods, iodine values of fatty acids and low-temperature crystallization techniques being utilized.

EXPERIMENTAL

From each of the normal five subjects in the post-absorptive state (8–10 hr.) about 250 ml. of blood was drawn via the antecubital vein into heparinized syringes. The blood was transferred to a sterile bottle to which had been added 50 ml. of a solution containing 3·0% of sodium citrate, 0·85% of sodium chloride and 2·0% of sodium sulphathiazole. Afterwards the whole solution was lyophilized and the bottle kept evacuated.

The ages of the above-mentioned five subjects were from 30 to 35 years. Their calorific and fat intakes per day per man were 2500–2640 and 55–65 g. respectively.

Another seven normal healthy men selected were from 33 to 40 years of age. Their calorific and fat intakes per day per man were 2268–2400 and 43–71 g. respectively. From each of the seven subjects in the post-absorptive state, about 40 ml. of blood was taken in a bulb containing sodium oxalate. Plasma and cells were separated as described by Evans *et al.* (1956).

Extraction of the lipid material. About 60 g. of lyophilized blood was treated with 600 ml. of ethanol at 60°. Ethanol was then distilled *in vacuo*. To the residue about 600 ml. of ethanol–ether (3:1) was added and warmed to 60°. It was kept overnight and then filtered. The treatment with ethanol–ether (3:1) was repeated until the lipids were completely extracted. To the whole ethanol–ether extract, 2 ml. of ethanolic 0·1% quinol was added and the mixture was evaporated on the water bath. Quinol was then washed out with hot water. The extract was treated with light petroleum (40–60°) and the lipids were recovered. The lipids from blood cells and plasma were also extracted in the same way as described above. The extracted lipid material was made to a volume of 100 ml. with light petroleum (40–60°) and portions were then taken for further analysis.

Analyses of lipids. Free and total cholesterol, total phospholipids and total fatty acids were estimated according to methods previously reported (Patil & Magar, 1959). The saturated fatty acid content was determined by oxidation of the fatty acids (Crombie, Comber & Boatman, 1955) in whole blood, and with blood cells and plasma it was calculated as the difference between the total fatty acids and the sum of oleic acid and polyunsaturated fatty acids. Wijs' iodine value was found by the semi-micro method of Sims & Stone (1956). Low-temperature crystallization of mixed fatty acids was carried out at −60° from acetone and at −25° from ether, according to the scheme of Gupta & Hilditch (1951). Polyunsaturated fatty acids were estimated by the alkali-isomerization spectrophotometric method of Herb & Riemenschneider (1953). It was assumed that the pentaenoic acids consisted of 50% of docosapentaenoic acid and 50% of eicosapentaenoic acid.

RESULTS AND DISCUSSION

Lipids and component fatty acids of plasma and blood cells. The levels of total lipids, total cholesterol and total fatty acids in blood cells were lower than those of plasma but the level of total phospholipids was high compared with that of plasma (Table 1). Ester cholesterol was much higher than free cholesterol in plasma. On the contrary, free cholesterol was higher than ester cholesterol in blood cells. This results in the level of free cholesterol in blood cells being higher than that of plasma, whereas the level of ester cholesterol was very low in blood cells as compared with that of plasma. This observation supports the findings of Erickson *et al.* (1937) and Brun (1939).

Iodine values of the fatty acids of plasma and blood cells varied from 102·3 to 106·2 and from 92 to 104·0 respectively. Saturated fatty acids comprised one-third of the total fatty acids in plasma and saturated fatty acids of blood cells constituted about half of the total fatty acids (Table 2). The unsaturated fatty acid fraction of plasma constituted 64–70% of the total fatty acids; oleic acid was the main component. In blood cells, the total unsaturated fatty acids contained about 50%, oleic acid being the major component. The level of oleic acid in blood cells was somewhat lower than that of plasma (Table 2). In the polyunsaturated fatty acids group, dienoic acid was the major component in plasma, whereas tetraenoic acid was the major component in blood cells. The level of dienoic acid in blood cells was quite low as compared with that of plasma (Table 2). The levels of pentaenoic acid and hexaenoic acid in blood cells and plasma were low, although there was more of both acids in the blood cells. Trienoic acid was present in small quantities in plasma, whereas it was absent from blood cells. Evans *et al.* (1956) were also unable to show the presence of trienoic acid in blood cells.

Low-temperature crystallization of fatty acids of lyophilized whole blood. Iodine values of whole-blood lipids varied from 73 to 75·9 and its mixed fatty acids from 102·0 to 112·0. By low-temperature crystallization at −60° and −25° in acetone and ether respectively, the mixed fatty acids were resolved into four fractions, differing in unsaturation (Table 3). The compositions of the fractions were studied by oxidation of the fatty acids, alkali-isomerization and determination of the iodine values. The fraction D contained predominantly higher polyunsaturated fatty acids. The fraction C, which contained saturated fatty acids, oleic acid and unsaturated fatty acids, was again recrystallized at −25° in ether. In the fraction B (soluble at −25°), the percentage of saturated fatty acids was decreased and the percentage of

Table 1. *Component lipids of plasma and blood cells of seven men*

Average values, ±S.D., are given.

Component lipid	Plasma (mg./100 ml. of plasma)	Blood cells (mg./100 ml. of packed cells)
Total lipid	485±11·6	397·7±12·6
Phospholipid	168±12·0	230·8±6·6
Free cholesterol	33±5·4	96·3±7·1
Ester cholesterol	127±9·4	21·3±3·0
Total cholesterol	160±7·7	117·6±6·6
Total fatty acids	343±7·4	238·7±8·0

Table 2. *Component fatty acids in plasma and blood cells of seven men*

Average values of percentage of total fatty acids, ±S.D., are given.

Component acids	Plasma	Blood cells
Saturated fatty acids	31·43±2·89	50·2 ±1·44
Oleic acid*	33·3 ±2·25	21·2 ±2·11
Dienoic acid	22·0 ±1·49	6·73±0·85
Trienoic acid	2·35±0·15	—
Tetraenoic acid	6·38±0·35	14·3 ±0·54
Pentaenoic acid	1·19±0·07	3·01±0·17
Hexaenoic acid	2·46±0·10	4·46±0·64

* Contained small amounts of palmitoleic acid.

Table 3. *Low-temperature crystallization of fatty acids of human whole blood*

A, Major group of saturated fatty acids; B, major group of monoethenoid fatty acids; C, saturated fatty acids, oleic acid and unsaturated fatty acid; D, major group of polyunsaturated fatty acids.

Fractions	Weight (g.)	Percentage (w/w)	Iodine value
A Insoluble at −25°	0·32±0·07	40·4±7·6	65·5±10·1
B Soluble at −25°	0·22±0·06	29·7±8·7	119·5± 9·0
C Insoluble at −60°	0·54±0·07	—	88·8± 5·5
D Soluble at −60°	0·23±0·06	29·9±7·2	156·6±10·7

Table 4. *Component lipids in lyophilized human whole blood*

	Average±S.D.
Blood lyophilized (g.)	60·7 ±2.2
Total lipids (g.)	1·29±0·06
Iodine value of lipids	74·7 ±1·2
Total fatty acids (g.)	0·77±0·03
Iodine value of fatty acids	109·0 ±2·3
	Concn. (g./100 g. of whole blood)±S.D.
Free cholesterol	0·19±0·01
Ester cholesterol	0·67±0·01
Total cholesterol	0·85±0·01
Phospholipids	0·96±0·04

Table 5. *Component fatty acids of human whole blood*

Acids	Percentage of total fatty acids±S.D.
Saturated	35·87±1·84
Oleic*	30·91±1·16
Dienoic	18·95±1·32
Trienoic	1·59±0·12
Tetraenoic	8·38±0·77
Pentaenoic	1·56±0·16
Hexaenoic	2·82±0·34

* Included small amounts of palmitoleic acid.

monoethenoid and other unsaturated fatty acids was increased. Fraction A (insoluble at −25°) consisted of large amounts of saturated fatty acids.

Lipids and component fatty acids of whole blood. Table 4 shows the range of variation in the percentage of cholesterol and phospholipids and total fatty acids in five samples of lyophilized whole blood. The cholesterol was chiefly in the ester form. The weight of phospholipids in whole blood was more than the total amount of free and ester cholesterol.

The percentage of oleic acid was highest in the unsaturated fatty acid group (30·9%), although the oleic acid contained a little palmitoleic acid which was not separately determined (Table 5). The total amount of polyunsaturated fatty acids comprised about one-third of the total fatty acids. In the polyunsaturated fatty acids, the dienoic acid was predominant although tetraenoic acid was also present in high concentrations. Trienoic acid, pentaenoic acid and hexaenoic acid were present in only small quantities. The levels of dienoic acid, trienoic acid and oleic acid were slightly lower than those of the plasma, whereas the levels of tetraenoic acid, pentaenoic acid, hexaenoic acid and saturated fatty acids were higher than those of plasma.

SUMMARY

1. The composition of lipids from plasma, blood cells and whole blood has been studied.

2. Saturated fatty acids and tetraenoic acids of blood cells were higher than those of plasma. The levels of oleic acid and dienoic acid of plasma were high compared with those of blood cells.

3. Oleic acid and polyunsaturated fatty acids of whole blood comprised each about one-third part of total fatty acids. The dienoic acid was the highest in the polyunsaturated fatty acids group.

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The Partial Synthesis of 6-Oxo-Oestriol and 6'α'-Hydroxyoestriol

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Mueller & Rumney (1957) showed that when [16-^{14}C]oestradiol-17β was incubated under aerobic conditions with mouse-liver microsomes and reduced triphosphopyridine nucleotide a number of radioactive metabolic products more 'polar' than oestradiol-17β were formed. One of these proved to be identical with 6-oxo-oestradiol-17β (Longwell & Wintersteiner, 1940); a second was shown to be identical with an authentic preparation of a 6-hydroxyoestradiol-17β supplied by Dr O. Wintersteiner which was described as '6β-hydroxyestradiol-17β'; while a third was identified as '6β-hydroxyestrone' since on reduction with sodium borohydride it yielded '6β-hydroxyestradiol-17β' and on oxidation it yielded 6-oxo-oestrone. Since the publication of this work Wintersteiner & Moore (1959) have given reasons for believing that the 6-hydroxyl group in the 6-hydroxyoestradiol-17β which is formed by metal-hydride reduction of 6-oxo-oestradiol-17β may have a quasi-equatorial configuration; accordingly these authors suggest that the reference compound supplied to Mueller & Rumney should be tentatively described as 6'α'-hydroxyoestradiol-17β. Breuer, Nocke & Knuppen (1958, 1959) have detected what are apparently the same 6-hydroxy oestrogen metabolites as were described by Mueller & Rumney among the products formed on incubating rat-liver slices with oestradiol-17β or oestrone.

No 6-oxo or 6-hydroxy oestrogen metabolites have yet been shown with certainty to occur in human urine, but unpublished and as yet incomplete work by K. H. Loke and G. F. Marrian has suggested that the Kober chromogen in the urine of pregnant women, which was designated KC-6B and which is similar in its chromatographic behaviour to 18-hydroxyoestrone in the Bush-type solvent systems used (Loke, Watson & Marrian, 1957; Loke, Marrian & Watson, 1959), may be a 6-hydroxyoestrone. The possibility that human urine might contain 6-oxo and 6-hydroxy derivatives of oestriol was therefore considered. Accordingly, as a preliminary to examining the urine of pregnant women for these compounds, 6-oxo-oestriol and 6'α'-hydroxyoestriol (terminology of Wintersteiner & Moore, 1959) were prepared from oestriol by the methods used for the preparation of 6-oxo-oestradiol-17β and 6'α'-hydroxyoestradiol-17β from oestradiol-17β by Longwell & Wintersteiner (1940) and Wintersteiner & Moore (1959).

EXPERIMENTAL AND RESULTS

Methods

Melting points of acetates were determined on a microscope hot-stage: those of the unacetylated compounds were determined in sealed evacuated capillaries. All melting points were determined on the same thermometer and are uncorrected for emergent stem.

Samples for C and H analysis, optical-rotation determinations and determinations of ultraviolet spectra were dried to constant weight at 100° *in vacuo*.

Optical rotations were determined in a 0·5 dm. tube with a diameter of 2·5 mm.

6-Oxo-oestriol triacetate

Crude oestriol (1·96 g.), prepared from late-pregnancy urine, was acetylated with acetic anhydride and anhydrous pyridine at room temperature for 24 hr., and the product recrystallized once from hexane. The recrystallized oestriol triacetate (1·87 g.) was dissolved in 6·5 ml. of acetic acid, and to this solution was added 1·7 g. of CrO_3 dissolved in 9·7 ml. of acetic acid and 1·4 ml. of water. After standing at room temperature for 24 hr. the excess of CrO_3 was reduced by the addition of 2 ml. of ethanol, and, after dilution with 200 ml. of water, the mixture was extracted with ether. The extract was washed with 50 ml. portions of saturated aq. $NaHCO_3$ until the washings showed a pink colour, then with 50 ml. of aq. 5 % (w/v) Na_2CO_3 saturated with $NaHCO_3$, and finally with 50 ml. of water. After being dried over Na_2SO_4 the ether solution of the non-acidic oxidation products was evaporated to dryness.

By treatment of this material (1·00 g.) with trimethylammonium hydrazide chloride in ethanolic acetic acid overnight at room temperature the ketonic fraction was obtained in the usual way. After reacetylation of the product, decolorization with charcoal in methanolic solution and recrystallization from methanol, 0·24 g. of crystalline material, m.p. 135–138°, was obtained.

This product was combined with 0·49 g. of similarly prepared material, and after crystallization again from methanol 0·57 g. of crystals, m.p.

137–139°, $[\alpha]_D^{16.5} - 41°$ in ethanol (*c*, 0·493), was obtained (Found: C, 67·3; H, 6·5. Calc. for $C_{24}H_{28}O_7$: C, 67·3; H, 6·6%).

The ultraviolet-absorption spectrum in ethanolic solution showed maxima at 249 mμ (ε 9150) and 298 mμ (ε 2260) and closely resembled that given for 6-oxo-oestradiol-17β diacetate by Longwell & Wintersteiner (1940).

6-Oxo-oestriol

6-Oxo-oestriol triacetate (110 mg.) was hydrolysed with 2·0 ml. of 20% (w/v) KOH in methanol for 24 hr. at room temperature in an atmosphere of N_2. After dilution with 30 ml. of water the solution was acidified to phenolphthalein with CO_2 and extracted three times with 10 ml. portions of ethyl acetate. After two washings with 5 ml. portions of water the extract was dried over Na_2SO_4 and evaporated to dryness. Crystallization of the product twice from methanol–benzene yielded 39 mg. of crystals, m.p. 240–242° (slight yellowing) and $[\alpha]_D^{16} - 7°$ in ethanol (*c*, 0·504) (Found: C, 71·5; H, 7·1. Calc. for $C_{18}H_{22}O_4$: C, 71·5; H, 7·3%).

The ultraviolet-absorption spectrum in ethanolic solution showed maxima at 257 mμ (ε 7895) and 325 mμ (ε 3052) and closely resembled that given for 6-oxo-oestradiol-17β by Longwell & Wintersteiner (1940).

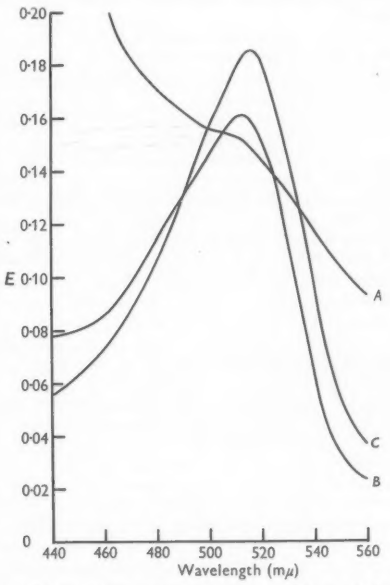

Fig. 1. Kober colour reactions on: *A*, 60·0 μg. of 6-oxo-oestriol; *B*, 4·03 μg. of 6'α'-hydroxyoestriol; *C*, 4·06 μg. of oestriol. Final vol. of reaction mixture, 3·3 ml.

The Kober reaction carried out by the method of Brown (1952) and Bauld (1954), with the former's 'oestriol reagent', was essentially negative (Fig. 1 *A*).

6'α'-Hydroxyoestriol

A solution of 84·5 mg. of 6-oxo-oestriol in 8·0 ml. of methanol was added dropwise to a solution of 70·7 mg. of $NaBH_4$ in 6·5 ml. of methanol. After standing for 2 hr. at room temperature the solution was acidified to litmus with 10% (w/v) aq. acetic acid and evaporated nearly to dryness at room temperature under an air stream. To the residue was added 3 ml. of water; the suspended crystals were filtered off with suction, washed with water and dried *in vacuo*.

Some difficulty was experienced in crystallizing the product, but eventually it was found that by evaporating a solution in 50% (v/v) aq. methanol slowly to just under half the original volume well-formed needles separated on cooling at room temperature. These were filtered off with suction, washed with water and dried *in vacuo*. The recrystallized product had m.p. 242–245° with evolution of gas (change of form at 140–150°) and $[\alpha]_D^{14} + 84°$ in ethanol (*c*, 0·498) (Found: C, 71·4; H, 7·6. Calc. for $C_{18}H_{24}O_4$: C, 71·0; H, 7·9%).

The ultraviolet-absorption spectrum in ethanolic solution showed a single maximum at 282 mμ (ε 2415) and closely resembled those of oestradiol-17β, oestrone and oestriol.

In the Kober reaction the spectral characteristics of the colour produced were normal (Fig. 1, *B*), but the extinction at the absorption maximum was only 63% of that given by the same weight of oestriol under the same conditions.

6'α'-Hydroxyoestriol tetra-acetate

About 40 mg. of recrystallized 6'α'-hydroxyoestriol (m.p. 244–248°) was acetylated in the usual way with acetic anhydride and pyridine at room temperature for 24 hr. The crude acetate was purified by filtering in benzene solution through a short column of alumina (acid-washed; activated at 100°) and crystallizing twice from methanol. The product had m.p. 159–161° and $[\alpha]_D^{16} - 12°$ in ethanol (*c*, 0·506) (Found: C, 66·2; H, 6·8. Calc. for $C_{26}H_{32}O_8$: C, 66·1; H, 6·8%).

Partition of oestriol, 6-oxo-oestriol and 6'α'-hydroxyoestriol between organic solvents and water

A chance observation made when 6-oxo-oestriol was first prepared by hydrolysis of its acetate suggested that this compound might be much less readily extracted from aqueous solution by ether than oestriol. In view of this the partition of oestriol, 6-oxo-oestriol and 6'α'-hydroxyoestriol in the systems ether–water and ethyl acetate–water

Table 1. *Partition of oestriol, 6-oxo-oestriol and 6'α'-hydroxyoestriol in the systems ether–water and ethyl acetate–water*

$$K = \left(\frac{\text{concn. in organic phase}}{\text{concn. in aqueous phase}}\right)$$

	Ether–water	Ethyl acetate–water
Oestriol	7·7	24
6-Oxo-oestriol	0·66	5·6
6'α'-Hydroxyoestriol	0·12	0·90

was determined quantitatively in the following way:

The steroid (100–300 μg.) spread in a thin film on the inside of a flask was shaken at 25° with 10 ml. of each phase (pre-equilibrated) until all had dissolved. The mixture was then transferred to a separating funnel and, after separation of the phases, 6·0 ml. of each was removed and evaporated to dryness in a vacuum desiccator. Each residue was dissolved in 3·0 ml. of ethanol and the concentration of steroid determined from the extinction of the solution at 281 mμ (oestriol and 6'α'-hydroxyoestriol) or 325 mμ (6-oxo-oestriol). The results (Table 1) show that the substitution of either an oxo or an 'α'-hydroxyl group at C-6 in oestriol has a surprisingly great effect in increasing the relative solubility in water.

DISCUSSION

Although neither 6-oxo-oestriol nor 6'α'-hydroxyoestriol has yet been shown to be present in the urine of pregnant women it is by no means improbable that they, or other highly oxygenated oestrogen derivatives with similar distribution characteristics in the systems ether–water and ethyl acetate–water, may be metabolic products of the oestrogenic hormone. In studies on the metabolism of radioactive oestrogens *in vivo* or *in vitro* it may therefore be dangerous to draw any conclusions about the nature of the metabolites formed (whether 'free', conjugated, or degraded to acidic substances) on the basis of the extractability of radioactive products from aqueous media by arbitrarily fixed proportions of ether or of other organic solvents (cf. Beer & Gallagher, 1955; Valcourt, Thayer, Doisy, Elliott & Doisy, 1955; Jellinck, 1959).

SUMMARY

1. 6-Oxo-oestriol, 6'α'-hydroxyoestriol and their acetates have been prepared from oestriol by methods based on those of Longwell & Wintersteiner (1940) and Wintersteiner & Moore (1959) for the preparation of 6-oxo-oestradiol-17β and 6'α'-hydroxyoestradiol-17β from oestradiol-17β.

2. Determinations of the distribution of 6-oxo-oestriol, 6'α'-hydroxyoestriol and oestriol in the systems ether–water and ethyl acetate–water showed that the substitution of an oxo or hydroxy group at C-6 in the oestriol molecule has a very great effect in increasing the relative solubility of these derivatives in water.

We gratefully acknowledge the help of Dr J. W. Minnis, who carried out the microanalyses.

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The Sedimentation of Myosin

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A wide range of reported values for the sedimentation coefficient of myosin at infinite dilution, S^0_{20} (termed henceforth the extrapolated sedimentation coefficient), exists in the literature. Values of 7·3 (Snellman & Tenow, 1948), 7·1 (Weber, 1950), 7·1 (Johnson & Landolt, 1951), and 6·7s (Mommaerts & Parrish, 1951) were earlier reported. More recently values of 6·38 and 6·43 (Holtzer & Lowey, 1956), 6·15 (Laki & Carroll, 1955) and 6·20s (Miller, Golder, Eitelman & Miller, 1952) have been obtained, the last two values referring to low temperatures. Moreover, the work of Laki & Carroll (1955) and Parrish & Mommaerts (1954) has suggested that the observed value may be a

function of the conditions, particularly the temperature and centrifugal field under which it is measured.

The work here reported was designed to investigate in detail the effects of varying temperature, centrifugal field and other experimental conditions on the extrapolated sedimentation constant. In addition to the experiments reported in which the rotor temperature and centrifugal field have been systematically varied, the effect of the buffer solution used, and of method of preparation, including the number of reprecipitations given to the sample, has also been investigated. It is shown that with certain important but understandable exceptions the extrapolated sedimentation constant for myosin is reproducible and independent of the conditions of measurement. A preliminary explanation of the anomalies previously observed and those now reported is given in terms of the molecular transformation of the myosin sample during the course of examination.

MATERIALS AND METHODS

Materials

Water. Water used was thrice-distilled in an attempt to remove traces of metal ions and possible surface-active material. A preliminary distillation was followed by two further distillations from Pyrex glass, the first in the presence of permanganate.

Reagents. All reagents used were of A.R. quality.

Buffer solution. The stock buffer solution used, with a pH of 6·7 and ionic strength I of 0·60, had the following composition (g./l.): KCl, 41·0; $Na_2HPO_4,12H_2O$, 4·0; KH_2PO_4, 2·2. All experiments were performed in this solution except where otherwise stated.

Myosin. Myosin was prepared from rabbit skeletal muscle by the method of Szent-Gyorgyi (1945), with several minor modifications. After killing and exsanguination, the back muscles were quickly excised and chilled in ice before mincing. All subsequent steps were performed near 0°. The muscle mince was extracted for 10 min. in Guba–Straub solution (0·3 M-KCl, 0·15 M-phosphate, pH 6·5), with gentle stirring. After removal of residual mince either by centrifuging or straining through muslin, the extract was filtered under slight pressure through a $\frac{3}{4}$ in. pad of paper pulp on a Büchner funnel. Especial care was taken to avoid frothing at this stage. The filtrate was then diluted to I 0·04, left standing and the supernatant was decanted; the precipitated myosin was brought into solution at I 0·5 with solid or 2·4 M-KCl, and allowed to stand for 1 hr. to ensure complete solution. After dilution to I 0·28 the small amount of actomyosin present was removed by centrifuging. After two or three reprecipitations at I 0·04, the myosin was dialysed against stock buffer solution and stored at 2–4° in sealed sterile flasks without preservative. Solutions thus obtained were clear, free from 'threads' of denatured protein, and showed a slight opalescence in bulk. No lipid material was noticed in any of the preparations, which is attributed to the pulp filtration stage (cf. Rupp & Mommaerts, 1957).

Methods

Sedimentation studies. Ultracentrifuge runs were performed in a Spinco Model E analytical ultracentrifuge equipped with diagonal-schlieren optics and facilities for continuous temperature measurement and control. Where necessary the rotor was pre-cooled or pre-heated to the desired operating temperature, and the automatic control used to maintain the temperature. Not only was the absolute calibration of the thermistor checked repeatedly against a standard (N.P.L.) thermometer, but the indicated temperature of the rotor in the rotor chamber at the end of an experiment was checked on the external calibrating stand as soon as the rotor was removed from the chamber. This procedure was adopted because it had been found in earlier experiments that appreciable resistance could be built up at or near the mercury-pool contact, giving a temperature error of up to 2°. By frequent renewal of the mercury pool, such errors were avoided.

The absolute accuracy of the final temperature measurements was normally $\pm 0{\cdot}05°$. In all runs the speed of the instrument was obtained by timing the revolution counter over a long interval. Except when otherwise stated, runs were performed at about 59 780 rev./min. and the centrifugal fields reported refer to the inner index position of the cell, assumed to be 5·725 cm. from the axis of rotation at 59 780 rev./min.

A cell with a dural centrepiece was employed for most experiments. Duplicate experiments with a plastic (Kel-F) centrepiece gave identical results, both as regards sedimentation coefficients and measurements of refraction difference. After measuring (on a Pye two-dimensional travelling microscope) the displacement of the maximum boundary ordinate on successive exposures taken at known time intervals, the sedimentation coefficients were evaluated from a plot of $\log x$ against time, where x is the distance from the axis of rotation. The mean magnification factor was calculated for each run by measuring the distance between the reference lines on the photographs. The coefficients obtained were corrected to the viscosity and density of water at 20° in the usual way (Svedberg & Pedersen, 1940). A constant value (0·728) for the partial specific volume, v (Parrish & Mommaerts, 1954), has been assumed over the range 2–26°. After most of this work was complete, $\bar{v}$ was independently determined by pyknometer (10 ml.) at 20° and found to be $0{\cdot}725 \pm 0{\cdot}005$. The relative viscosity, obtained by capillary viscometer to $\pm 0{\cdot}001$, and density of the stock buffer solution were determined at 20°, 10° and 5°. Viscosity was calculated for other temperatures by assuming the relative viscosity, $\eta_{rel.}$, to be independent of temperature over the interval of interpolation. Since $\eta_{rel.}$ has the values 0·991, 0·988 and 0·977 at 20°, 10° and 5° respectively, no significant error is involved in this procedure.

Concentration measurements. Nitrogen estimation by the micro-Kjeldahl method (Pregl & Roth, 1945) has been used as a primary standard, by taking a value of 16·2% for the nitrogen constant of myosin (Mommaerts & Parrish, 1951). For routine determinations, a modified biuret estimation has been employed (Rowe, unpublished work). This has been checked against the primary standard for each preparation. Over the range of protein concentration used in this work, it was confirmed that repeated transfers of a protein solution between glass vessels did not cause a

significant decrease in protein concentration (cf. Rupp & Mommaerts, 1957).

Area measurements. The areas of schlieren peaks in the sedimentation diagrams were measured by enlarging and tracing the diagrams, and then estimating the enlarged areas either by counting squares or by cutting out the tracings and weighing relative to a standard area; for the latter method, good-quality airmail paper was found to be sufficiently uniform.

Diffusion coefficients. Apparent diffusion coefficients (B), better designated as 'spreading constants' (Parrish & Mommaerts, 1954), have been calculated by the 'height–area' method, correcting both total area and maximum height for the fall of concentration during centrifuging.

RESULTS

Sedimentation under varying conditions at $17 \pm 2°$

All freshly prepared samples gave rise to a single well-defined sedimenting boundary in the ultracentrifuge (Fig. 1, i–iv). More rapidly sedimenting components appeared after storage (Fig. 1, v), as first noted by Weber (1950). Almost all sedimentation runs have therefore been performed within ten days of preparing the sample. Fig. 2 contains collected sedimentation coefficients for several myosin preparations over a range of protein concentration and solvent composition. Table 1 shows the high reproducibility of the extrapolated sedimentation coefficients from four preparations. The regression line, calculated statistically from all the data at $17 \pm 2{\cdot}0°$, is given by

$$S^0_{20} \text{ (in Svedberg units, s)} = 6{\cdot}43\ (\pm 0{\cdot}04) - 4{\cdot}3\ (\pm 0{\cdot}3)c \quad (1)$$

where c is the concentration in g./100 ml. Standard deviations are given in parentheses. The results obtained in a Kel-F centrepiece are not significantly different (cf. Blum & Morales, 1953).

Effect of nature of solvent. Experiments were performed with three different, commonly-employed solvents: (i) 0·5M-KCl; (ii) 0·6M-KCl; (iii) 0·55M-KCl buffered with phosphate to a total ionic strength I of 0·60. Fig. 2 illustrates the results obtained, the regression line being obtained

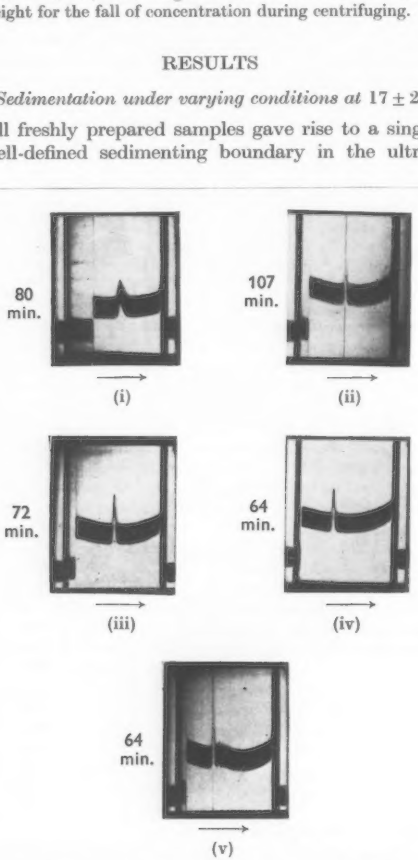

Fig. 1. Sedimentation diagrams for various myosin preparations [in stock buffer solution except (i)]: (i) Myosin I in 0·5M-KCl at 0·156 g./100 ml. and 44 770 rev./min.; (ii) myosin III, 0·31 g./100 ml.; (iii) myosin IV, 0·146 g./100 ml.; (iv) myosin V, 0·17 g./100 ml.; (v) myosin III, as (ii) but stored for 34 days at 2–4°. Approximate times of sedimentation are given alongside the sedimentation diagrams.

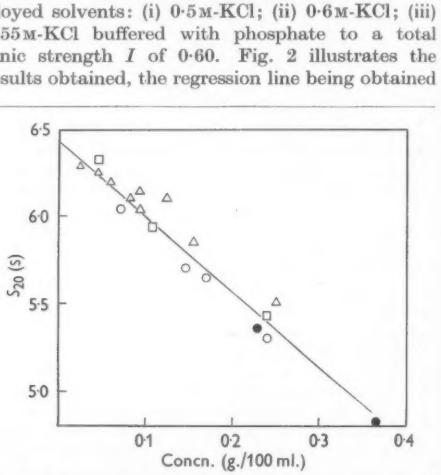

Fig. 2. Sedimentation coefficient (expressed in s) of myosin at 17° in various solvents. △, 0·5M-KCl; □, 0·6M-KCl; ○, stock buffer solution (KCl–phosphate); ●, stock buffer solution and Kel-F centrepiece.

Table 1. *Extrapolated sedimentation coefficient,* S^0_{20}, *for different myosin preparations in stock buffer solution*

Preparation	Number of reprecipitations	S^0_{20} (s)
I	3	6·41*
III	2	6·52
IV	2	6·36
V	0	6·41

* Includes data from experiments in 0·5M- and 0·6M-KCl.

by the method of least squares, and including all points. It is apparent that any effect of the varying electrolytes must, if present, be small, and certainly less than that required to explain the different values found in the literature.

Effect of reprecipitation of myosin. Snellman & Gelotte (1951) reported for 'once-crystallized' myosin a value for the extrapolated constant of 7·2s, whereas for heart myosin and twice-precipitated myosin the values of S_{20} at definite concentrations were noticeably lower. A straight-line extrapolation for the latter data (not given by Snellman & Gelotte) would yield a value of about 6·5s for the extrapolated constant. The difference was attributed to a lower actin content in the twice-precipitated sample.

Since it seemed possible that the number of reprecipitations employed could influence the observed sedimentation coefficient, a sample of once-precipitated myosin was prepared and examined. A plot of observed S_{20} values against concentration yielded a value of 6·41s for the extrapolated coefficient, and a slope not significantly different from that given by equation (1). In Table 1 this result can be compared with the values obtained for other samples, whose preparation involved 1–3 reprecipitations. No significant effect of reprecipitation can be discerned.

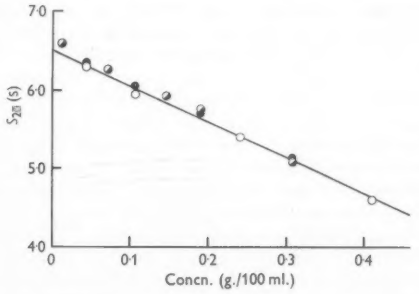

Fig. 3. Sedimentation coefficients (s) of myosin in stock buffer solution at various temperatures: ○, 18±2°; ●, 9±1°; ◐, 4±2°.

Table 2. *Effect of rise in temperature on the sedimentation coefficient at various concentrations*

Preparation	Concn. (g./100 ml.)	Corr. sedimentation coeff. (s) 17°	25°
III	0·24	5·31*	5·95
	0·143	5·70*	5·87
IV	0·356	4·86	5·38
	0·181	5·66	5·89
VIII	0·292	5·16	5·09
	0·146	5·80	5·70

* By interpolation from S–c curve.

Sedimentation at 2–10°

Fig. 3 illustrates the results obtained for the sedimentation of a myosin preparation at 4° and 9°, compared with those obtained at room temperature. When the size of the correction factors (up to 80% for the lowest temperatures) is taken into account, the agreement between corrected S_{20} values for the three temperatures is excellent. It may be concluded that over this range of temperature, and under these conditions, no significant effect of temperature upon the corrected sedimentation coefficient of myosin exists. This view has been confirmed at single concentration values for other preparations. Only with one preparation was an anomalous value, $S^0_{20} = 7{\cdot}1$s at 9°, obtained. There is no certain explanation for this single discrepancy, thought it may be significant that this preparation was unusually labile, as judged by the time required for the appearance of faster-sedimenting components.

Sedimentation at 24–26°

Anomalously high sedimentation coefficients were obtained over this range for certain preparations (Table 2).

The increases in S_{20} obtained with preparations III and IV, though not large, are significant. Considerable variation from one preparation to another is evident, and the data for any particular preparation were in general less consistent than those obtained at lower temperatures.

Sedimentation at different centrifugal fields

Experiments were performed at constant protein concentration with speeds varying from 20 420 to 59 780 rev./min., giving a ninefold variation in centrifugal field. Parrish & Mommaerts (1954) originally reported that the effect of centrifugal field on S_{20} was greatest at higher temperatures. Experiments were therefore performed at 25±1° in order to accentuate any effect which might be found.

Fig. 4 illustrates typical results obtained. A definite effect of field on observed sedimentation coefficient, especially marked at fields below 100 000 g ($\omega^2 = 2 \times 10^7$), is evident. Experiments with two other preparations confirmed the very considerable effect at low fields and the almost negligible effect at fields in excess of 100 000 g.

Effect of molecular transformation

It is seen from the above data that the extrapolated sedimentation constant of myosin is a well-defined parameter under a range of conditions at

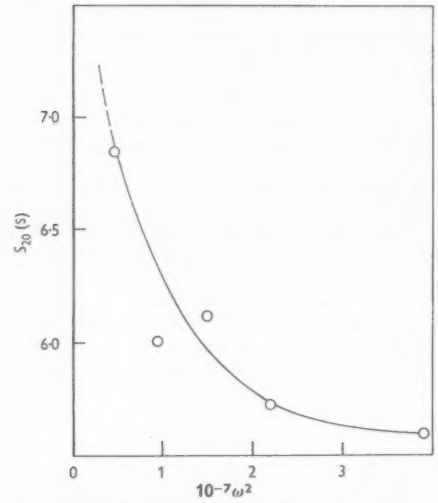

Fig. 4. Sedimentation coefficient (s) of a sample of myosin in stock buffer solution as a function of centrifugal field (concn., 0·146 g./100 ml.; temp., 25±1°).

temperatures below 20°. At higher temperatures increased values are obtained, the increase being small and variable in amount at high centrifugal fields, but pronounced at low centrifugal fields. These conclusions are substantially in accordance with the more recent data published by Mommaerts & Aldrich (1958). The question arises whether the anomalous behaviour at the higher temperatures can be explained in terms of the known properties of myosin, without postulating a dependence on temperature or centrifugal field of the sedimentation coefficient of the native protein.

Since it is known (Weber, 1950; Spicer & Weise, 1955; Holtzer, 1956) that myosin can give rise to species sedimenting more rapidly, and that the velocity of this reaction is strongly temperature-dependent, an explanation in terms of this myosin transformation seems possible. There are two extreme sets of conditions under which the formation of components of high sedimentation coefficient could give rise to the observed effects in a solution which initially contains unaltered myosin.

(1) Under conditions of high resolution for the system (that is with maximal centrifugal field and path length in the cell), faster components would become separated from the main sedimenting boundary and travel ahead.

A fall in protein concentration at the main sedimenting boundary would result, causing more rapid sedimentation than would correspond to the initial total myosin concentration. Experimental points would therefore lie above the normal S_{20}–c curve. Some consequences of these conditions applying are: (*a*) Since the transformation into faster components must be continuous throughout the run, the formation of a discrete faster boundary would not necessarily be expected; rather there would be a slight raising of the base line in front of the main peak in the schlieren diagram, which might or might not be detectable. (*b*) The corrected areas of myosin peaks as observed in the schlieren diagram should decrease in magnitude during the course of a run at higher temperatures. (*c*) The observed increase in corrected sedimentation coefficient over low-temperature values should be small, and in no case should values greater than the extrapolated low-temperature constant be obtained. (*d*) Calculated values for S_{20} should increase continuously during a run. (2) Under conditions of low resolution for the system (with small centrifugal fields and short path length in the cell), different consequences follow: (*a*) Interdiffusion of the myosin and transformed components would maintain a constant concentration (apart from radial dilution) at the broad sedimenting boundary, and this would be reflected in the constancy of corrected peak areas measured from sedimentation diagrams. (*b*) The position of the maximum ordinate of the resultant schlieren peak would be a complex function of the relative concentration, diffusion coefficients and concentration dependence of the kinetic properties of the various components present. (*c*) Although the different components of the boundary would not be resolved under these conditions, and the corrected schlieren peak area would remain constant, the change in composition should give rise to a boundary spreading more rapid than could be accounted for in terms of the diffusion coefficient of native myosin. This might, however, be obscured by the boundary-sharpening process arising from the concentration-dependence of the sedimentation constant. (*d*) The values for S_{20} should again tend to increase during a run, and could conceivably be higher than the extrapolated low-temperature constant.

However, at both high and low resolutions anomalous effects would be noticeable only at higher temperatures, when an appreciable quantity of myosin is transformed into faster-sedimenting materials during the time taken by an experiment. Then at high centrifugal fields a small increase in S_{20} over low-temperature values would be obtained, while at low fields a much larger effect would be expected. The longer runs required at low fields would make the latter effects even more readily observable.

Experimental data are in detailed agreement with the predictions made above (Figs. 5–8; Table 3). At higher temperatures and high centri-

fugal fields, the areas of the schlieren peaks decrease during a run, an effect not shown at low temperatures or with low fields (Fig. 5). The schlieren diagrams at 25° show distinct evidence of faster-sedimenting material ahead of the main peak; over the range 5–17° no such effect is noticeable (Fig. 6). It was possible to estimate the concentration of unaltered myosin from the mean peak area during a sedimentation experiment and, on plotting S_{20} against corrected concentration, results at 25° are in good agreement with those at lower temperatures (cf. Fig. 7*a*, *b*). Thus the dynamic anomaly reported by Parrish & Mommaerts (1954) is quantitatively explained.

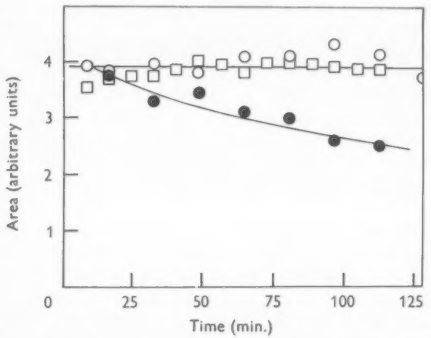

Fig. 5. Schlieren peak area, corrected for radial dilution of myosin (0·146 g./100 ml.), sedimenting in stock buffer solution under various conditions. ○, Temp. 24·6°, $\omega^2 = 0{\cdot}9 \times 10^7$; ●, temp. 20°, $\omega^2 = 3{\cdot}9 \times 10^7$; □, temp. 2·2°, $\omega^2 = 3{\cdot}9 \times 10^7$.

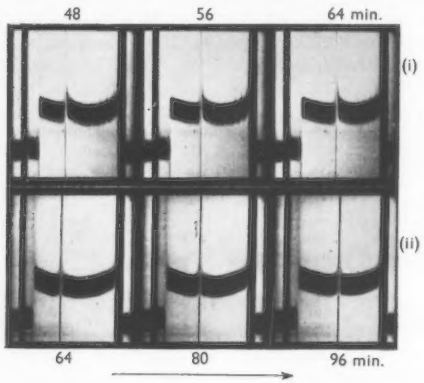

Fig. 6. Sedimentation diagrams for myosin in stock buffer solution at (i) 23·5°, (ii) 17·9°, concn., 0·41 g./100 ml. The approximate time of sedimentation is given for each diagram.

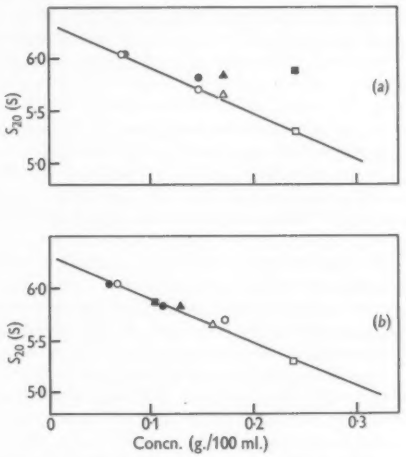

Fig. 7. Sedimentation coefficient (s) of myosin at 17±2° (open symbols) and at 25±1° (closed symbols) plotted as a function of concentration derived from (*a*) Kjeldahl nitrogen estimation, (*b*) peak-area measurements. Solvents: ○ and ●, stock buffer; △ and ▲, 0·6 M-KCl; □ and ■, 0·5 M-KCl.

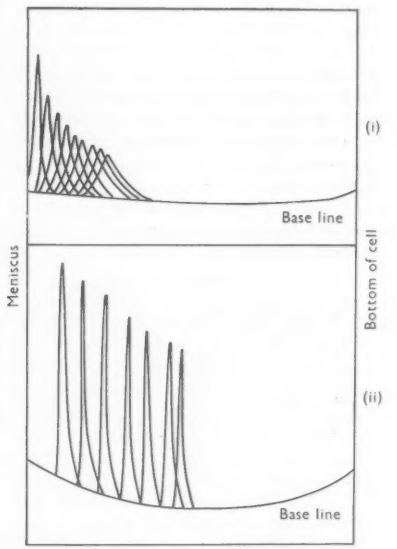

Fig. 8. Boundary spreading during ultracentrifuging of myosin solutions: (i) temp. 24·6°, 0·146 g./100 ml., $\omega^2 = 0{\cdot}9 \times 10^7$; (ii) temp. 4·5°, 0·17 g./100 ml., $\omega^2 = 3{\cdot}9 \times 10^7$. Enlarged tracings correspond to 16 min. intervals.

In Fig. 8, boundary spreading at 24° and low speed is compared with that at high speed and low temperature. Although in both cases the total area corrected for radial dilution at the boundary remains constant, the apparent rate of diffusion is found to be considerably greater in the former case ($B_{20} = 9{\cdot}02 \times 10^{-8}$ c.g.s. unit) than in the latter ($B_{20} = 6{\cdot}5 \times 10^{-8}$ c.g.s. unit), indicating the occurrence of pronounced boundary spreading due to increasing polydispersity at the higher temperature.

Table 3 illustrates the change in sedimentation rate during the course of a run. As would be expected, this is significant only for the low-speed run at high temperature. Because of the inevitable time of standing of the solution during evacuation of the rotor chamber and acceleration of the rotor, together with the need to consider a range of several exposures in order to obtain a reliable value for the sedimentation coefficient, only about a third of the total expected change could be studied by this method. Since at 59 780 rev./min. even the most labile preparations seldom gave an S^0_{20} greater than the low-temperature value by more than 0·3 s (see Table 2), an increase of 0·1 s (at the most) could be expected during the course of a high-speed run. Much more appreciable increases were, however, observed at low speed.

It remained to confirm that: (*a*) the rate of formation of transformed products from myosin was such that an appreciable proportion of the protein would be transformed during the time required for a sedimentation run; (*b*) the difference in sedimentation coefficient between myosin and its transformation products was sufficient to account for the observed behaviour. Such confirmation has been obtained in a detailed study of the heat-transformation reaction of myosin (unpublished work). The reaction has been found to follow first-order kinetics over much of its course, and reaction constants have been evaluated for various conditions. Table 4 gives values for half-life times, $t_{\frac{1}{2}}$, for various preparations at different temperatures (columns 2 and 3), and column 4 gives the calculated percentage decrease in myosin concentration occurring in 70 min. at 25° as a result of the transformation reaction. Because of the finite time needed for resolution of faster components, the actual dilution at the boundary must in all cases be less than the maximal values quoted.

The variation in stability between different preparations is noticeable (compare last columns of Table 4 with Table 2). Sedimentation coefficients have usually been evaluated at a mean time corresponding to about 70 min. after insertion of the sample into the cell. Bearing this in mind, the values for $t_{\frac{1}{2}}$ in Table 4 confirm that no effect would be expected at room temperature (17°) or below, and that with a particularly stable preparation, such as myosin VIII, little effect would be discernible at high speeds, the lowered concentration at the sedimenting boundary (even at 25°) being insufficient to give rise to a measurable rise in S_{20}.

It has been suggested (S. Lowey, personal communication) that the presence of phosphate in the buffer causes retardation of the transformation reaction, but in the present work no such effect could be confirmed. Thus the percentage transformation in stock buffer at 25° in 2 hr. of a myosin solution containing 0·24 g./100 ml. was 24, as against 22 in 0·6 M-KCl.

The heat transformation of myosin regularly gives rise to two distinct faster-sedimenting species, peak II and peak III (see Fig. 9). The relative amounts of components II and III are estimated as about two parts of component II to three parts of component III. Component III appears to be the more polydisperse of the two. Fig. 10 shows the

Table 3. *Changes of sedimentation rate during sedimentation*

Concn. of protein, 0·2 g./100 ml. t_1, t_2 represent the times after placing ice-cold solution in the cell at the temperatures given in the third column.

Preparation	Centrifugal field (*g*)	Temp.	t_1 (min.)	S_{20} (s)	t_2 (min.)	S_{20} (s)
III	228 000	25·2°	33	5·83	123	5·85
VIII	26 700	24·8°	33	5·96	305	6·26

Table 4. *Half-life times ($t_{\frac{1}{2}}$) and allied data for the myosin transformation reaction in stock buffer solution*

Preparation	$t_{\frac{1}{2}}$ (min.) 17°	$t_{\frac{1}{2}}$ (min.) 25°	Calc. (70 min.) decrease in concn. at 25° (%)	S_{20} at 25° and 59 780 rev./min.
III	1156	100	38	High
IV	605	65	53	High
VIII	1800	184	23	Normal

sedimentation coefficients of the two components as a function of protein concentration. In each case the concentration taken has been that of the total protein at the sedimenting boundary, estimated from peak-area measurements. Both components sediment more rapidly than myosin, and their presence even at low concentration is adequate to explain the observed increases in S_{20}.

DISCUSSION

The sedimentation coefficient of myosin at infinite dilution is reproducible under a variety of conditions, including a wide range of temperature if allowance is made for conversion of myosin into faster-sedimenting species. The value of

$$S^0_{20} = 6{\cdot}43 \pm 0{\cdot}04\text{s}$$

reported is in good agreement with those reported by Holtzer & Lowey (1956) of 6·38 and 6·43s, and that of Hippel, Schachman, Appel & Morales (1958) of $6{\cdot}25 \pm 0{\cdot}15$s for the slow component in '5-hr. actomyosin' (i.e. from extraction of muscle mince with Weber–Edsall solution for 5 hr.). All these values are much lower than those originally proposed for myosin (Snellman & Tenow, 1948; Weber, 1950; Johnson & Landolt, 1950, 1951). It seems probable that the difference is to be accounted for in terms of the transformation reaction, since the earlier values were obtained under conditions of lower resolution (i.e. lower centrifugal field) and higher temperature than those obtaining in recent studies; the sedimentation diagrams of Snellman & Tenow (1948) suggest the presence of appreciable amounts of transformed material. The formation of an appreciable proportion of transformed material during a run would then be expected to cause a high sedimentation coefficient (cf. Table 2).

The value $S^0_{20} = 6{\cdot}66$s reported by Parrish & Mommaerts (1954), which requires correction (Waugh & Yphantis, 1952) for the adiabatic cooling of the rotor (giving 6·83s), is also significantly higher than that reported here, even though it was determined under conditions of high resolution comparable with those employed in the present work. However, the temperature employed was $23{\cdot}6 \pm 1{\cdot}5°$ and, though no quantitative estimate of the amount of transformation can be made, it seems highly probable that this would be appreciable and sufficient to account for the high sedimentation constant reported.

Three reports have suggested that near 0° a low value for S^0_{20} is obtained (Miller *et al.* 1952; Laki & Carroll, 1955; Mommaerts & Aldrich, 1958). These authors obtained values of 6·20s at 7°, 6·15s at 4° and 6·10–6·20s at 6° respectively, though in no case were detailed results given over a range of concentrations. In the present investigation, no evidence has been found that the corrected sedimentation coefficient is in any way affected by lowered temperature. In reaching this conclusion, particular care has been exercised in confirming the accuracy and reproducibility of the temperature measurements on the ultracentrifuge (p. 433), and it seems quite feasible that faulty operation of the thermistor assembly might be responsible for some of the discrepancies now apparent. The recent suggestion by Mommaerts & Aldrich (1958) that the correct value for S^0_{20} might lie between 5·8 and 6·0s is, in the authors' opinion, without foundation.

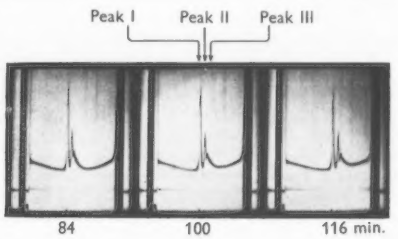

Fig. 9. Sedimentation diagrams for a myosin solution incubated in stock buffer for 183 min. at 25° before sedimentation at 18–20° and 59 780 rev./min. Protein concn., 0·40 g./100 ml. The approximate time of sedimentation is given for each diagram.

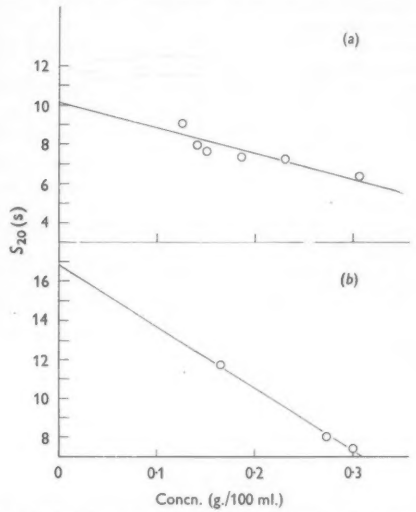

Fig. 10. Sedimentation coefficients (s) of myosin-transformation products [peak II (*a*) and peak III (*b*) are as in Fig. 9]. Concentrations are expressed as total protein concentration at the sedimenting boundary.

Assuming the value 6·43s for the extrapolated sedimentation coefficient, it is clear that even with Weber's (1950) diffusion coefficient ($0{\cdot}9 \times 10^{-7}$ c.g.s. unit) and his other data, the molecular weight becomes 740 000, considerably smaller than had been earlier visualized. However, it seems likely that the myosin transformation reaction would cause lowered diffusion coefficient as well as raised sedimentation coefficient and the more recent value ($1{\cdot}05 \times 10^{-7}$) of Parrish & Mommaerts (1954) is probably to be preferred. With $\bar{v} = 0{\cdot}725$ this gives a molecular weight of 540 000. This value is in substantial agreement with light-scattering results, but is considerably larger than recent values obtained by the Archibald technique.

The reported 'dynamic anomalies' of Parrish & Mommaerts (1954) have been largely confirmed, so far as an apparent effect of higher temperatures and lowered centrifugal fields has been demonstrated. However, such anomalies arise, not from peculiar hydrodynamic properties of the myosin molecule, but from the occurrence of a reaction in which myosin is transformed into faster-sedimenting species in a time which is comparable with that required for a sedimentation-velocity experiment. The agreement between the predicted consequences of such a transformation occurring during sedimentation and the experimental results observed is excellent, within the limits of the present qualitative approach; and there now seems to be little reason for postulating 'dynamic anomalies' in the sedimentation behaviour of native, untransformed myosin solutions.

SUMMARY

1. The sedimentation of rabbit myosin has been investigated under conditions of varying temperature, centrifugal field and solvent composition.

2. From 4° to 19° the corrected sedimentation coefficient is reproducible over a range of solvent composition, and its variation with concentration is given by

$$S^0_{20}\,(\mathrm{s}) = 6{\cdot}43\,(\pm 0{\cdot}04) - 4{\cdot}3\,(\pm 0{\cdot}3)c.$$

Variation in the number of precipitations of the myosin had no observable effect on the sedimentation behaviour.

3. At 25° the occurrence of previously reported apparent anomalies has been confirmed. These are small at high centrifugal fields, but relatively large at low fields.

4. Evidence is presented to show that these anomalies may be explained in terms of the transformation of myosin into faster-sedimenting species, without the necessity for assigning unusual hydrodynamic properties to the myosin molecule.

5. With

$$S^0_{20} = 6{\cdot}43\mathrm{s}, \quad D^0_{20} = 1{\cdot}05 \times 10^{-7}, \quad \bar{v} = 0{\cdot}725,$$

the calculated molecular weight is 540 000.

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Effect of Dietary Fat and Cholesterol on the Polyunsaturated Fatty Acids of Cholesterol Esters, Phospholipids and Triglycerides in the Liver of the Rat

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Garton & Duncan (1957) found that the cholesterol in the plasma of animals was combined mainly as the linoleate ester, so the relation of dietary fats with or without cholesterol to the component lipids of liver and plasma and to the levels of polyunsaturated fatty acids of liver- and plasma-lipid fractions has become an important subject of investigation. Alfin-Slater, Aftergood, Wells & Deuel (1954) reported that there was an accumulation of cholesterol esters in the livers of polyunsaturated-fatty acid-deficient rats. Klein (1958) observed that the liver cholesterol esters were increased in fat deficiency, were low in normal ranges of dietary fat and increased again with high levels of linoleic acid feeding. Klein (1958) and Okey, Lyman, Harris, Einset & Hain (1959) observed that esterified linoleic acid in liver was directly related to dietary linoleic acid. Thus it is reasonable to expect that an analysis of the fatty acid composition of each lipid class will yield information about the transport and metabolism of fatty acids.

In this paper results of the investigation of the effect of dietary fats with and without cholesterol on the polyunsaturated fatty acids of liver cholesterol esters, phospholipids and neutral glycerides are reported.

EXPERIMENTAL

Animals and diet. These results were obtained as part of a study of the relation of dietary fat and cholesterol to tissue and serum cholesterol and polyunsaturated fatty acids in rats (Patil & Magar, 1959). Estimations of polyunsaturated fatty acids were carried out on the cholesterol esters, phospholipids and neutral glycerides found in the livers of male albino rats maintained on diets containing 5% of coconut oil, dalda (hydrogenated fat), ghee (butter fat) or groundnut oil. The other ingredients of the diet were 22% of casein, 4% of salt mixture and 69% of sucrose. The rats were given all the required vitamins in sufficient quantities (Cheng, Alfin-Slater & Deuel, 1954). After 6 weeks on the above-mentioned diets the six rats of each group received 0·5 g. of cholesterol/kg. body wt. The cholesterol was mixed in the respective oils, which were then mixed with the diets. After 10 months of feeding on cholesterol, all rats were killed.

Extraction of lipids and analysis. The fresh liver tissue was homogenized in 10% (w/v) trichloroacetic acid solution containing 0·4 M-$MgCl_2$ and the lipids were then extracted and purified by the method of Johnson & Dutch (1951). The phospholipids were precipitated with acetone from a known quantity of the lipid, and the acetone-soluble fraction and acetone-insoluble fractions were purified and weighed accurately. About 600–700 mg. of acetone-soluble lipids were chromatographed on silicic acid (Merck) by the method of Fillerup & Mead (1953) as modified by Mead & Fillerup (1954), 6·4 g. of activated silicic acid being used for 50 mg. of acetone-soluble lipids. Cholesterol esters were eluted first, followed by glycerides and free sterols. These fractions were evaporated *in vacuo*, dried and weighed. The weighed quantities of phospholipids, cholesterol esters and glycerides were saponified for the isolation of fatty acids according to the procedure of Wiese & Hansen (1953). The fatty acids were isomerized and analysed spectrophotometrically by the method of Herb & Riemenschneider (1953). Calculations of the percentage of the individual polyunsaturated fatty acids were made according to Herb & Riemenschneider (1953), as modified by Hammond & Lundberg (1953), for the inclusion of hexaenoic acid. In the formulae used in the present work, it was assumed that the pentaenoic acids consisted of 50% of docosapentaenoic acid and 50% of eicosapentaenoic acid.

RESULTS AND DISCUSSION

Liver lipids. The composition of component lipids of the liver in rats fed with diets containing 5% of fats with and without cholesterol is given in Table 1. The percentage of lipids accumulated in the liver of rats fed with diets containing 5% of various fats plus cholesterol was significantly higher than those of animals fed with fats alone. Table 1 shows that the amount of phospholipid in liver lipids from animals fed with diets containing 5% of various fats with cholesterol was largely reduced as compared with those values for animals fed only with fats. On the other hand, cholesterol esters and triglycerides were high in cholesterol-fed groups as compared with those of groups not fed with cholesterol. Liver phospholipids were slightly higher, whereas liver cholesterol esters and triglycerides were slightly lower in rats fed on groundnut oil with and without cholesterol as compared with those fed on the other three fats with and without cholesterol.

Table 1. *Composition of liver lipids of rats fed on diets containing 5% of various fats with and without cholesterol*

Figures represent the average values for six rats ± S.D.

Diet	Lipids (% of liver wt.)	Component lipids as percentage of total lipids			
		Phospholipids	Sterol esters	Triglycerides	Free sterols
Coconut oil	8·0 ± 0·7	42·9 ± 2·5	1·5 ± 0·1	53·0 ± 2·0	2·6 ± 0·2
Coconut oil + cholesterol	11·1 ± 1·1	18·2 ± 1·8	13·5 ± 0·9	65·5 ± 2·7	2·8 ± 0·2
Dalda	7·8 ± 0·6	43·1 ± 2·8	1·4 ± 0·2	52·9 ± 2·1	2·6 ± 0·1
Dalda + cholesterol	11·2 ± 0·8	21·0 ± 1·5	14·1 ± 1·0	62·1 ± 1·8	2·8 ± 0·2
Ghee	7·5 ± 0·6	46·5 ± 2·2	1·4 ± 0·1	49·4 ± 2·5	2·7 ± 0·2
Ghee + cholesterol	10·4 ± 0·8	25·0 ± 1·5	11·8 ± 0·8	60·2 ± 2·3	3·0 ± 0·2
Groundnut oil	6·9 ± 0·5	54·3 ± 2·1	1·2 ± 0·1	41·7 ± 1·6	2·8 ± 0·2
Groundnut oil + cholesterol	10·1 ± 0·7	30·1 ± 2·0	10·0 ± 0·7	57·0 ± 2·2	2·6 ± 0·2

Table 2. *Polyunsaturated fatty acids in liver cholesterol esters of rats fed on diets containing 5% of various fats with and without cholesterol for 10 months*

Figures represent the average values for six rats ± S.D.

Diet	Polyunsaturated fatty acids as percentage of total cholesterol ester fatty acids				
	Dienoic	Trienoic	Tetraenoic	Pentaenoic	Hexaenoic
Coconut oil	6·43 ± 0·35	1·15 ± 0·08	2·20 ± 0·32	0·12 ± 0·03	0·8 ± 0·07
Coconut oil + cholesterol	4·12 ± 0·28	2·30 ± 0·09	1·24 ± 0·28	0·00	0·58 ± 0·07
Dalda	6·34 ± 0·23	1·82 ± 0·12	2·34 ± 0·26	0·18 ± 0·02	0·92 ± 0·08
Dalda + cholesterol	3·87 ± 0·32	2·10 ± 0·06	1·13 ± 0·27	0·00	0·71 ± 0·06
Ghee	6·94 ± 0·25	1·92 ± 0·12	2·26 ± 0·30	0·20 ± 0·04	1·1 ± 0·05
Ghee + cholesterol	5·19 ± 0·32	2·51 ± 0·10	1·41 ± 0·30	0·00	0·8 ± 0·04
Groundnut oil	13·2 ± 0·34	1·00 ± 0·07	3·52 ± 0·31	0·23 ± 0·04	1·41 ± 0·04
Groundnut oil + cholesterol	10·4 ± 0·51	1·50 ± 0·08	2·00 ± 0·34	0·00	0·97 ± 0·03

Table 3. *Polyunsaturated fatty acids in liver phospholipids of rats fed on diets containing 5% of various fats with and without cholesterol for 10 months*

Figures represent the average values for six rats ± S.D.

Diet	Polyunsaturated fatty acids as percentage of total phospholipid fatty acids				
	Dienoic	Trienoic	Tetraenoic	Pentaenoic	Hexaenoic
Coconut oil	8·72 ± 0·73	0·85 ± 0·12	13·6 ± 1·2	2·51 ± 0·36	2·10 ± 0·32
Coconut oil + cholesterol	6·25 ± 0·53	1·40 ± 0·16	9·42 ± 1·1	1·82 ± 0·31	1·28 ± 0·21
Dalda	8·93 ± 0·65	0·55 ± 0·08	13·2 ± 1·3	2·72 ± 0·30	2·52 ± 0·22
Dalda + cholesterol	6·11 ± 0·55	1·12 ± 0·10	10·4 ± 1·1	2·12 ± 0·25	1·82 ± 0·21
Ghee	8·56 ± 0·91	0·64 ± 0·06	12·8 ± 0·9	2·67 ± 0·32	2·22 ± 0·18
Ghee + cholesterol	6·21 ± 0·62	1·72 ± 0·13	10·1 ± 0·8	1·09 ± 0·24	1·81 ± 0·31
Groundnut oil	12·3 ± 0·71	0·31 ± 0·05	17·7 ± 1·1	3·20 ± 0·31	2·97 ± 0·20
Groundnut oil + cholesterol	10·1 ± 0·78	0·67 ± 0·08	14·6 ± 1·0	2·41 ± 0·33	2·32 ± 0·20

Table 4. *Polyunsaturated fatty acids in liver triglycerides of rats fed on diets containing 5% of various fats with and without cholesterol for 10 months*

Figures represent the average values for six rats ± S.D.

Diet	Polyunsaturated fatty acids as percentage of total triglyceride fatty acids				
	Dienoic	Trienoic	Tetraenoic	Pentaenoic	Hexaenoic
Coconut oil	5·21 ± 0·65	4·23 ± 0·35	2·61 ± 0·28	0·24 ± 0·06	0·30 ± 0·05
Coconut oil + cholesterol	3·94 ± 0·51	6·40 ± 0·40	2·30 ± 0·31	0·21 ± 0·03	0·21 ± 0·03
Dalda	5·56 ± 0·50	3·95 ± 0·51	2·65 ± 0·32	0·75 ± 0·07	0·25 ± 0·04
Dalda + cholesterol	3·38 ± 0·45	6·93 ± 0·62	2·54 ± 0·32	0·71 ± 0·05	0·22 ± 0·03
Ghee	5·39 ± 0·62	3·75 ± 0·40	2·41 ± 0·25	0·61 ± 0·06	0·12 ± 0·03
Ghee + cholesterol	3·98 ± 0·45	7·65 ± 0·62	2·40 ± 0·21	0·60 ± 0·07	0·12 ± 0·04
Groundnut oil	7·90 ± 0·70	1·57 ± 0·32	2·91 ± 0·36	1·41 ± 0·05	0·82 ± 0·04
Groundnut oil + cholesterol	7·22 ± 0·65	2·85 ± 0·40	2·81 ± 0·31	1·28 ± 0·05	0·71 ± 0·04

Polyunsaturated fatty acids of cholesterol esters, phospholipids and triglycerides. The individual polyunsaturated fatty acids of cholesterol esters, phospholipids and triglycerides of liver lipids of animals fed with fats with and without cholesterol are represented in Tables 2, 3 and 4 respectively. In animals fed with fats only, dienoic acids appeared to be the major group in both the cholesterol esters and phospholipid fractions, occurring to the extent of 6·3–13·2% and 8·5–12·3% respectively. The level of this acid was low (5·2–7·9%) in the triglyceride fraction. Trienoic acid occurred in the largest quantities in the triglycerides (1·6–4·2%) and in the lowest amounts in the phospholipids (0·3–0·8%). Table 3 shows that the liver phospholipids contained tetraenoic acid, pentaenoic acid and hexaenoic acid in greater proportion than those present in the liver cholesterol esters (Table 2) and triglycerides (Table 4). It is clear from Tables 2 and 4 that the distribution of polyunsaturated fatty acids in the triglycerides was similar to that of cholesterol esters.

Effect of dietary fats on polyunsaturated fatty acids of liver cholesterol esters, phospholipids and triglycerides. The percentages of dienoic acid and tetraenoic acid in the liver phospholipids and the levels of dienoic acids in the cholesterol esters of animals fed with a diet containing 5% of coconut oil, hydrogenated fat or butter fat were observed to be lower (Tables 2 and 3) than those of animals fed with a diet containing 5% of groundnut oil. Klein (1958) found a high percentage of dienoic acid (45·5 mg./100 mg. of total fatty acids) in cholesterol ester fatty acids in the livers of rats fed with diets containing 81·2 mg. of linoleic acid/g. of diet. Also, Okey *et al.* (1959) obtained 16–27% and 13–21% of dienoic acid from liver cholesterol ester fatty acids in rats fed with diets containing 10% of corn oil and 10% of groundnut oil respectively. In the present experiments the comparatively low levels of dienoic acids in the cholesterol esters, phospholipids and triglycerides were due to the low percentage of dietary fat, and the small amounts of linoleic acid contained in coconut oil, hydrogenated fat, butter fat (2·0–2·5%) and groundnut oil (26·2%) as compared with the linoleic acid contained in corn oil (50–60%).

Table 2 suggests that the tetraenoic acids were low in the liver cholesterol esters of animals fed on coconut oil, hydrogenated fat, butter fat or groundnut oil. This is in conformity with the results of Klein (1958), which showed low levels of tetraenoic acid in liver cholesterol esters of animals fed on diets containing 30% of corn oil.

The levels of tetraenoic acid, pentaenoic acid and hexaenoic acid were very low in the triglycerides. Trienoic acid was high and dienoic acid was low in the liver triglycerides of animals fed on coconut oil, hydrogenated fat or butter fat as compared with those of animals fed on groundnut oil. Mead & Slaton (1956) expressed the opinion that the trienoic acid in liver may be 5:8:11-eicosatrienoic acid and not linolenic acid. This trienoic acid was shown by Montag, Klenk, Hayes & Holman (1957) to be structurally related to oleic acid.

Effect of feeding with cholesterol on polyunsaturated fatty acids of liver cholesterol esters, phospholipids and triglycerides. The levels of dienoic acids were significantly decreased in the liver phospholipids (Table 3), cholesterol esters (Table 2) and triglycerides (Table 4) of animals fed on fats with cholesterol as compared with those fed with fats alone. The levels of dienoic acid were decreased in liver phospholipids, cholesterol esters and triglycerides and the levels of trienoic acid were significantly increased in the triglycerides of animals fed with coconut oil plus cholesterol, hydrogenated fat plus cholesterol, butter fat plus cholesterol as compared with those of animals fed on groundnut oil plus cholesterol (Tables 2–4).

Pentaenoic acid was completely absent and tetraenoic acid and hexaenoic acid were lowered in the liver cholesterol ester of animals fed with cholesterol (Table 2). The levels of tetraenoic acid, pentaenoic acid and hexaenoic acid were reduced in the liver phospholipids as a result of cholesterol feeding (Table 3). The changes in the levels of tetraenoic acid, pentaenoic acid and hexaenoic acid in the liver triglycerides of cholesterol-fed animals were, however, very small.

SUMMARY

1. Liver phospholipids were reduced and cholesterol esters and triglycerides were increased as a result of feeding cholesterol to rats.

2. In rats fed with diets containing 5% of various fats for 10 months, the dienoic acid level was high in liver cholesterol esters and phospholipids. Tetraenoic acid level was low in the cholesterol esters and triglycerides and high in phospholipids. The liver phospholipids contained more tetraenoic acid, pentaenoic acid and hexaenoic acid than the cholesterol esters and triglycerides.

3. The levels of polyunsaturated fatty acids of liver lipids decreased greatly on feeding on more saturated fats with and without cholesterol as compared with those obtained when less saturated fats, with and without cholesterol, were fed.

4. Trienoic acids were increased on cholesterol feeding much more in the liver triglycerides than the cholesterol esters and phospholipids.

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Tissue-Fractionation Studies

14. THE ACTIVATION OF LATENT DEHYDROGENASES IN MITOCHONDRIA FROM RAT LIVER*

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Several mitochondrial enzymes are known to occur in a latent form, for example adenosine triphosphatase (Kielley & Kielley, 1951), glutamic dehydrogenase (Hogeboom & Schneider, 1953), aconitase (Dickman & Speyer, 1954), cytochrome *c* reductases (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955; Borgström, Sudduth & Lehninger, 1955), cytochrome oxidase (Simon, 1958), rhodanese (Greville & Chappell, 1959) and alkaline deoxyribonuclease (Baudhuin, 1959). The activities of the enzymes in intact mitochondria towards added substrates are relatively low and can be increased, sometimes tenfold or more, by damage to the particles. The acid hydrolases which are localized in the lysosomes of rat liver behave in a similar manner (de Duve *et al.* 1955). These enzymes can be liberated into solution and their activities revealed by various procedures such as incubation at 37°, freezing and thawing, treatment in a Waring Blendor, osmotic shock and treatment with detergents and certain enzymes (Berthet & de Duve, 1951; Berthet, Berthet, Appelmans & de Duve, 1951; Appelmans & de Duve, 1955; Gianetto & de Duve, 1955; Wattiaux & de Duve, 1956; Beaufay & de Duve, 1959). In graded activation experiments the activities of the various hydrolases appear concurrently. There is good evidence that the latency of lysosomal enzymes is due to the inability of the substrates to penetrate the membrane of the particle, and that the apparent activation is due to rupture of the membrane and release of the enzymes into solution.

This paper describes the results of a parallel study of the release of enzymes associated with the two types of particle. Acid phosphatase was taken as being a typical lysosomal enzyme and glutamic dehydrogenase was chosen to represent mitochondria. Some experiments were also done with malic and D-β-hydroxybutyric dehydrogenases. A brief account of the results has already been given (Bendall, 1958), and evidence that these dehydrogenases belong to the true mitochondria has been described (Beaufay, Bendall, Baudhuin & de Duve, 1959; Beaufay, Bendall, Baudhuin, Wattiaux & de Duve, 1959).

EXPERIMENTAL

Methods

All experiments were performed on a combined heavy and light mitochondrial fraction (which contains most of the lysosomes as well as the mitochondria) isolated in 0·25 M-sucrose from rat liver by the method described by Appelmans, Wattiaux & de Duve (1955). The particles were washed once, resuspended in 0·25 M-sucrose (at a concentration corresponding to 0·5–1·0 g. of original liver/ml.) and stored at 0° before use. In a few experiments the preparation and suspension medium contained mM-ethylenediaminetetra-acetic acid (EDTA), pH 7·7.

Acid phosphatase. Free acid phosphatase was measured as described by Gianetto & de Duve (1955). Total activities were determined by the addition of 0·1 % of Triton X-100

* Part 13: Beaufay, Bendall, Baudhuin, Wattiaux & de Duve, 1959.

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to the reaction medium as described by Wattiaux & de Duve (1956).

Glutamic dehydrogenase. In order to minimize the errors caused by changes in turbidity, free glutamic dehydrogenase was measured by following spectrophotometrically the reduction of cytochrome *c* at 550 mμ in the presence of purified reduced diphosphopyridine nucleotide (DPNH) cytochrome *c* reductase. This has two advantages over following the reduction of diphosphopyridine nucleotide (DPN); in the first place it is more sensitive by a factor of about 6, and secondly the turbidity correction is smaller at 550 mμ than at 340 mμ. For the measurement of free activity the final reaction mixture contained 0·02 M-glycylglycine–NaOH buffer, pH 7·7, 0·03 M-nicotinamide, 0·4 mM-sodium cyanide, mM-EDTA, 0·25 M-sucrose, 0·7 mM-DPN, 0·013 M-potassium L-glutamate, 80 μM-cytochrome *c*, 6 units of reductase and 0·05 ml. of enzyme in a total volume of 1·8 ml. The reaction was started by the addition of enzyme and the increase in extinction at 550 mμ and 25° was followed in a Beckman model D.U. spectrophotometer with a reference cell containing all components of the system except glutamate. For measurement of total activity the reaction mixture was the same but the particle preparation was diluted in a solution containing 0·1 % of Triton X-100 and 0·25 M-sucrose. This was preferred to the direct addition of Triton X-100 to the reaction medium since at this concentration the detergent caused some inhibition. Fig. 1 shows that the maximum activity obtained by this method is the same as that obtained by prolonged blender treatment, and it is therefore probable that it gives a true measure of the total activity. In general the sucrose concentration in the reaction medium was always 0·25 M since it caused some inhibition of glutamic dehydrogenase (about 25 %). The linear portions of progress curves were used for the calculation of rates. The rate of cytochrome *c* reduction sometimes fell a little at high particle concentrations; to correct for this a calibration curve was always constructed with three different concentrations of Triton-treated particles.

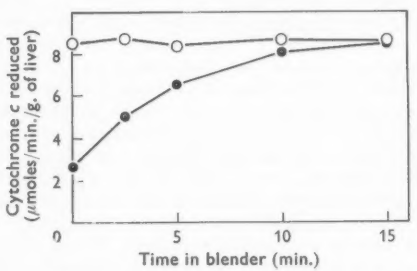

Fig. 1. Comparison of maximum activity of glutamic dehydrogenase obtained by treatment in blender and with Triton X-100. Mitochondria (1 g. of liver/ml.) suspended in 0·25 M-sucrose were diluted 10 times in cold water and treated for various lengths of time in a cooled MSE blender turning at full speed. Sucrose was added to bring the concentration to 0·25 M. Samples were diluted for assay (by the cytochrome *c* method) either in sucrose (●) or in sucrose containing 0·1 % of Triton X-100 (○).

The DPNH cytochrome *c* reductase used in these experiments was not very suitable because its relatively high Michaelis constants made it necessary to use high concentrations, and because of the instability of the purified enzyme in solution at 0°. In some later experiments glutamic dehydrogenase was assayed by following DPN reduction at 340 mμ with the same reaction mixture as described above with the omission of cytochrome *c* and reductase. Under these conditions the error caused by turbidity changes was found to be appreciable only when low free activities were being measured. The necessary correction was made by taking readings alternately at 340 and 410 mμ (where DPNH does not absorb). An experimental factor of 1·49 was used to convert E_{410} into E_{340}.

D-β-*Hydroxybutyric dehydrogenase.* This enzyme was assayed by the cytochrome *c* method described above. The reaction medium was the same as for glutamic dehydrogenase except that the substrate was 0·05 M-sodium DL-β-hydroxybutyrate. Only the D-isomer will have been oxidized since the L-isomer is oxidized only after conversion into the coenzyme A derivative (Lehninger & Greville, 1953), which could not have been formed under the experimental conditions used. In a few experiments the DPN method was also used. Triton X-100 and a number of other detergents inhibited the enzyme strongly. In order to obtain total activities a suspension in 0·25 M-sucrose was diluted 40 times in water and treated for 10 min. in a cooled blender (Measuring and Scientific Instruments Ltd.) turning at full speed.

L-*Malic dehydrogenase.* The enzyme was assayed by following the reduction of DPN at 340 mμ by the method described by Beaufay, Bendall, Baudhuin & de Duve (1959), except that 0·25 M-sucrose was added to the reaction medium and Triton X-100 was omitted. Total activities were determined by dilution of the particles in a solution containing 0·1 % of Triton X-100 and 0·25 M-sucrose.

Materials

Reduced diphosphopyridine nucleotide cytochrome c reductase. The enzyme was purified from pig heart up to the stage R_3 by the method described by Mahler, Sarkar, Vernon & Alberty (1952), with the difference that the first centrifuging was for 5 min. at 1000 *g* only. The final precipitate was dissolved in a 2 % (w/v) solution of crystalline bovine serum albumin and stored in small samples in the deep-freeze (−25° approx.). The enzyme was assayed by following spectrophotometrically the reduction of cytochrome *c* at 550 mμ and 25° in a total volume of 1·8 ml. containing 0·03 M-glycylglycine–NaOH buffer, pH 7·7, 80 μM-cytochrome *c*, 0·2 mM-DPNH and enzyme. The reference cell contained all components except enzyme. The unit of activity, defined as change in extinction of 1·00/min. under the above conditions, is slightly different in value from that defined by Mahler *et al.* (1952).

Reagents. Glycylglycine, DPN, cytochrome *c* and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) were obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.), Triton X-100 from Rohm and Haas Co. (Philadelphia, Pa., U.S.A.), L-glutamic acid from the Pfanstiehl Chemical Co. (Waukegan, Ill., U.S.A.), sodium DL-β-hydroxybutyrate from British Drug Houses Ltd., L-malic acid from Eastman Organic Chemicals (Rochester, N.Y.) and crystalline bovine plasma albumin from The Armour

Laboratories (Eastbourne). DPNH was prepared by enzymic reduction of DPN as described by de Duve *et al.* (1955).

RESULTS

The activities of the three mitochondrial dehydrogenases were very low in untreated preparations, usually less than 10% of the maximum. They were considerably increased by procedures which activate lysosomal enzymes, namely treatment with detergents, hypotonic conditions, freezing and thawing and mechanical disruption. In general, however, the two types of particle differed in their sensitivity to these treatments.

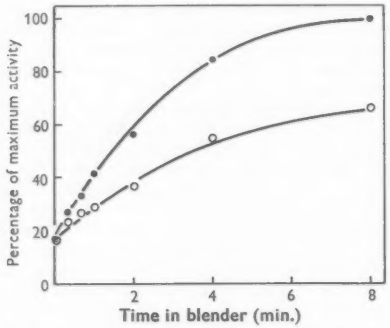

Fig. 2. Activation of glutamic dehydrogenase (●) and acid phosphatase (○) by blender treatment. Samples of mitochondrial fraction (1 g. of liver/5 ml.) suspended in 0·25 M-sucrose were treated for various lengths of time in a cooled MSE blender turning at low speed. Glutamic dehydrogenase was assayed by the cytochrome *c* method.

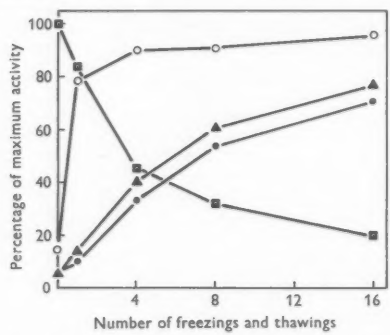

Fig. 3. Activation of glutamic dehydrogenase (●), β-hydroxybutyric dehydrogenase (▲) and acid phosphatase (○) by freezing and thawing. Samples of mitochondrial fraction (1 g. of liver/ml.) suspended in 0·25 M-sucrose in stoppered tubes were frozen in acetone–solid CO_2 and thawed in tap water. Dehydrogenases were assayed by the cytochrome *c* method. ■, Extinction of the suspension at 500 mμ plotted as a percentage of that of the untreated preparation. Extinction of the blender-treated sample used to obtain maximum activity of β-hydroxybutyric dehydrogenase was subtracted from that of the other samples before calculation of the percentage.

Mechanical disruption

Fig. 2 shows the activation of glutamic dehydrogenase and acid phosphatase by treatment of the particles in a blender for various lengths of time. In this experiment the mitochondrial enzyme was more readily activated than the lysosomal enzyme. However, rather variable results have been obtained with blender experiments and the reverse situation has been observed.

Freezing and thawing

Freezing and thawing gave a more clear-cut distinction between the two types of particle (Fig. 3). A few cycles of freezing and thawing gave almost complete release of acid phosphatase. On the other hand, glutamic and β-hydroxybutyric dehydrogenases were considerably more resistant, and even after 16 cycles there was only about 70% activation. In this experiment the decrease in turbidity as a result of freezing and thawing was determined by the change in extinction of the suspension at 500 mμ. The curve obtained showed a reciprocal relation with the activation curve of the mitochondrial enzymes, and clearly was not related to the activation curve of acid phosphatase.

Osmotic activation

Irreversible activation. The irreversible osmotic lysis of mitochondria is shown in Fig. 4. Samples of a particle preparation were diluted in sucrose solutions of decreasing concentration and were allowed to stand in these media for 15 min. at 0°. Sufficient sucrose was then added to return the concentration to 0·25 M and the free activities of the enzymes were determined as soon as possible. Acid phosphatase gave a gradual activation curve which is typical of lysosomes (Appelmans & de Duve, 1955; Gianetto & de Duve, 1955). Glutamic and β-hydroxybutyric dehydrogenases were much more resistant; appreciable activation did not occur until the sucrose concentration was as low as 0·025 M and below that concentration it occurred fairly sharply. The curves for the two dehydrogenases followed each other closely, except at very low sucrose concentrations, where considerably lower activities were obtained for β-hydroxybutyric dehydrogenase. This may be due either to a difference in the intramitochondrial location of the two enzymes, which is suggested by a difference in their solubilities (see below), or to some inactivation of β-hydroxybutyric dehydrogenase at low sucrose

concentrations. In this experiment, the turbidity of the suspension again decreased concurrently with the increase in activity of the dehydrogenases.

The results of a similar experiment are represented in Fig. 5, showing that the activation of malic dehydrogenase also closely follows that of glutamic dehydrogenase. The activities of malic dehydrogenase were actually a little lower but this was probably due to the differences in the conditions for assay of the two enzymes, in particular to the fact that glutamic dehydrogenase was measured by following cytochrome *c* reduction and malic dehydrogenase by following DPN reduction. Greville & Chappell (1959) have shown that the osmotic activation of rhodanese follows a very similar type of curve.

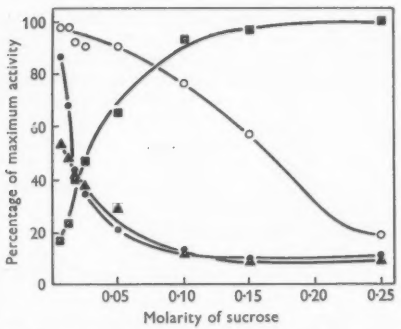

Fig. 4. Osmotic activation of glutamic dehydrogenase (●), β-hydroxybutyric dehydrogenase (▲) and acid phosphatase (○). Preparations were diluted in sucrose solutions of various concentrations. After 15 min. at 0° sucrose was added to bring the concentration to 0·25 M. Dehydrogenases were assayed by the cytochrome *c* method. ■, Extinction of the suspension at 500 mμ measured 9 min. after activation and plotted as a percentage as in Fig. 3.

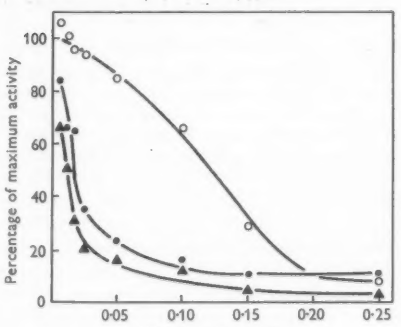

Fig. 5. Osmotic activation of malic dehydrogenase (▲), glutamic dehydrogenase (●) and acid phosphatase (○). Activation was as shown in Fig. 4. Glutamic dehydrogenase was measured by cytochrome *c* reduction and malic dehydrogenase by DPN reduction.

Reversible activation. Irreversible osmotic activation does not occur until the sucrose concentration is as low as 0·025 M. However, reversible osmotic swelling can occur at much higher concentrations (Tedeschi & Harris, 1955), and it was of interest to know whether a reversible osmotic activation of glutamic dehydrogenase occurs at the same time. To test this possibility it was necessary to do the preliminary activation in the reaction medium, except that DPN was not added until the reaction was started. The calculated osmolar concentration of the activation medium was brought to a minimum of 0·0362 M by reducing as much as possible the concentrations of the components of the reaction system, and it was varied by the addition of sucrose. After activation for 5 min. only at 25° the activity of glutamic dehydrogenase was determined either in this same medium with the sole addition of DPN, or after addition of sucrose to bring the concentration to 0·25 M. These results have been plotted in Fig. 6. There was a considerable degree of irreversible activation, which was greater than usual because of the higher temperature of activation. The small difference between the two curves shows that there was only a small degree of reversible osmotic activation.

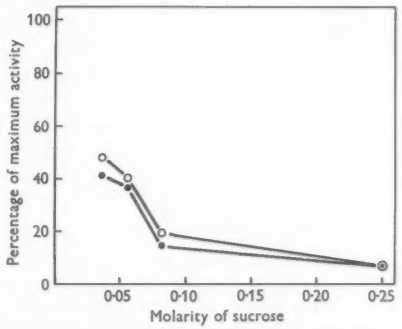

Fig. 6. Reversible osmotic activation of glutamic dehydrogenase. Activation was for 5 min. at 25° in a medium containing 0·01 M-glycylglycine–NaOH buffer, pH 7·7, neutralized 0·4 mM-NaCN, mM-EDTA, 80 μM-cytochrome *c*, pH 7·7, 3 units of DPNH cytochrome *c* reductase/ml., 5 mM-potassium glutamate or 5 mM-KCl (for the blank) and sucrose to bring the osmolar concentration to the value shown on the abscissa. The calculated osmolar concentration of the activation medium without sucrose was 0·0312 M. The reaction was started immediately after activation by the addition of 0·7 mM-DPN with (●) or without (○) the addition of 0·25 M-sucrose. Reduction of cytochrome *c* followed at 550 mμ and 25°.

Table 1. *Solubility of glutamic dehydrogenase after treatment in a blender*

Samples of mitochondria suspended in 0·25 M-sucrose containing mM-EDTA, pH 7·7, (1 g. of liver/2 ml. of suspension), were treated in a MSE blender for the times stated below, diluted twice in sucrose–EDTA and centrifuged for 30 min. at 100 000 *g* in a Spinco model L ultracentrifuge with rotor no. 40. Glutamic dehydrogenase was assayed by the cytochrome *c* method; activities are represented as the percentage of the total activity of the suspension before centrifuging.

Time in blender (min.)	Original suspension. Free	Supernatant. Total	Pellet Total	Pellet Free	Recovery
0	9·1	2·1	99·5	10·0	101·6
15	67·7	73·1	29·3	5·9	102·4
30	75·5	74·9	10·2	4·9	85·1

Table 2. *Solubility of glutamic dehydrogenase after treatment with Triton X-100*

Mitochondria were suspended in 0·25 M-sucrose containing mM-EDTA and various concentrations of Triton X-100 (mitochondrial concn.: 1 g. of liver/2 ml. of suspension). After standing for 15 min. at 0° samples were centrifuged and glutamic dehydrogenase activities were determined as described in Table 1. Figures represent percentages of the total activities of the suspensions before centrifuging (0·1 % of Triton did not cause complete activation because of the high concentration of particles).

Concn. of Triton X-100 (%)	Original suspension. Free	Supernatant. Total	Pellet. Total	Recovery
0	9·3	0·9	101·4	102·3
0·05	4·8	2·3	90·5	92·8
0·1	20·4	5·4	100·0	105·4
1·0	95·8	100	6·0	106·0

Table 3. *Solubility of malic and β-hydroxybutyric dehydrogenase after osmotic activation*

Activation in 0·00625 M-sucrose was carried out as described in Fig. 4. After transfer to 0·25 M-sucrose the free activity was measured on a sample and the remainder was centrifuged for 30 min. at 100 000 *g*. Activities are represented as percentages of the total activities of the original suspension. Assays were by DPN reduction.

	Malic dehydrogenase	β-Hydroxybutyric dehydrogenase
Free activity	73·1	46·5
Soluble activity	32·8	0·3
Particulate activity	81·2	51·0
Recovery	114·0	51·3

Solubility of the enzymes after activation

Activation of glutamic and malic dehydrogenases was found to be associated with a liberation of the enzymes into solution, but β-hydroxybutyric dehydrogenase remained bound to the insoluble residue of the particles. This was investigated by a comparison of the free activity of an activated suspension with the activities in the supernatant and in the pellet after centrifuging the suspension at 100 000 *g* for 30 min. in a Spinco preparative ultracentrifuge. The solubility of glutamic dehydrogenase after blender and Triton X-100 treatment is shown in Tables 1 and 2. In either case, when the free activity was high there was a good correlation between free activity and soluble activity. However, when the free activity was low it was considerably higher than the soluble activity.

Fig. 7. Correlation of free and soluble activities of glutamic dehydrogenase after osmotic activation. Activation was as in Fig. 4. The activated suspensions (after transferring to 0·25 M-sucrose) were centrifuged for 30 min. at 100 000 *g*. Soluble activity (●) was measured on the supernatant, and free activity (○) on the activated suspension. Assay was by DPN reduction.

The correlation between soluble glutamic dehydrogenase and free activity after hypotonic treatment is shown in Fig. 7. There was an appreciable liberation of enzyme into solution but the soluble activity was distinctly lower than the free activity. The results obtained with malic and β-hydroxybutyric dehydrogenases in the same experiment are shown in Table 3. Malic dehydrogenase behaved like glutamic dehydrogenase; some of the activity was liberated into solution but soluble activity was

lower than free activity. On the other hand, practically all the activity of β-hydroxybutyric dehydrogenase was recovered in the pellet. This result is not conclusive because of the poor recovery, but the insolubility of β-hydroxybutyric dehydrogenase is in agreement with the results of other workers. For example, the phosphorylating sub-mitochondrial particles which have been obtained by digitonin (Cooper & Lehninger, 1956) and sonic (McMurray, Maley & Lardy, 1958; Kielley & Bronk, 1958) treatment of mitochondria retain this enzyme but not glutamic dehydrogenase.

DISCUSSION

Four different treatments have been found to activate the latent glutamic dehydrogenase of mitochondria, in each case up to maximum values of approximately the same magnitude. In several instances, this activation could be correlated with changes in the turbidity of the mitochondrial suspension, with a certain degree of solution of the enzyme and with the simultaneous activation of two other latent dehydrogenases. These facts, together with the varied nature of the activating agents themselves, make it very likely that the latter all act by modifying a structural barrier restricting the accessibility of the enzymes to one or more of their substrates. It has been found by Lehninger (1951) that DPNH is oxidized very slowly by intact mitochondria and the latency of the dehydrogenases under the conditions of our assays is therefore explainable already on the basis of a relative impermeability of the mitochondrial membrane to DPN. However, it is probable that the penetration of the oxidizable substrates is also limited, in view of the low rates of oxidation of these substrates with molecular oxygen as final acceptor (Johnson & Lardy, 1958), as compared with the maximum activity of the dehydrogenases (Beaufay, Bendall, Baudhuin & de Duve, 1959) and of the electron-transport system (de Duve *et al.* 1955).

Several types of activation may be distinguished within the general framework of the above interpretation. The most clear-cut one goes together with a quantitative release of the enzyme molecules in soluble form, as is the case for glutamic dehydrogenase after blender treatment or exposure to high concentrations of Triton X-100. A more complex type is the one affecting glutamic dehydrogenase in the presence of low concentrations of Triton X-100 and both glutamic and malic dehydrogenases after exposure to media of low tonicity. Partial solution of the enzymes occurs under these conditions, but the free activities are higher than the soluble activities. This could be due to the fact that the mitochondrial membrane has been rendered more fragile by the activating procedure and that some of the particles burst when transferred from 0° to the reaction medium at 25°. Alternatively, it is possible that the damage suffered by some of the mitochondria is sufficient to allow the substrates to penetrate into the particles but not to allow the enzymes to leak out of them. That the latter phenomenon may occur, even to some extent in a reversible manner, is suggested by the results of Fig. 6 and by those of Ernster & Navazio (1956), who observed an activation of glutamic dehydrogenase by ageing under aerobic conditions at 30°, which could be reversed by the addition of adenosine triphosphate. It is likely from the work of Greville & Chappell (1959) that the activation of rhodanese is similar to that of the dehydrogenases. These authors have made the interesting observation that agents such as phosphate and thyroxine which induce swelling of mitochondria also cause an activation of rhodanese. The relation between activation and solubility will depend also on the type of link existing between the enzymes and the insoluble framework of the mitochondria. This link appears to be much stronger for β-hydroxybutyric dehydrogenase than for the other two dehydrogenases studied in this work.

Despite these complicating factors, the activation curves of the three mitochondrial enzymes showed a fairly close parallelism, and differed significantly in several cases from the corresponding curves obtained for the lysosomal acid phosphatase. These differences could possibly be due to the fact that acid phosphatase was assayed under more drastic conditions (pH 5·0, 37°) than the dehydrogenases (pH 7·7, 25°). However, this cannot be the only explanation, since the osmotic activation of acid phosphatase has been found to follow the same course, whether the enzyme is assayed at pH 5·0 and 37° or at pH 6·1 and 0° (Appelmans & de Duve, 1955). The results obtained in this investigation may therefore be taken as providing an independent line of evidence for the existence of mitochondria and lysosomes as distinct species of particle. In general, lysosomes appear to be more sensitive than mitochondria to osmotic rupture and to damage by freezing and thawing, but not necessarily to mechanical disruption in a blender.

SUMMARY

1. A comparison has been made between the activation of a latent mitochondrial enzyme glutamic dehydrogenase, and that of a lysosomal enzyme, acid phosphatase, by freezing and thawing, hypotonicity, the neutral detergent Triton X-100 and blender treatment.

2. Malic and D-β-hydroxybutyric dehydrogenases were also found to occur in mitochondria in

a latent form. The activation of these enzymes was compared with that of glutamic dehydrogenase.

3. In graded activation experiments the activation curves for the three dehydrogenases followed each other closely, but they were distinct from the curves for acid phosphatase.

4. A marked irreversible osmotic activation occurred at sucrose concentrations below 0·025 M. At higher sucrose concentrations only a small degree of reversible activation of glutamic dehydrogenase could be observed.

5. Activation led to a liberation of glutamic and malic dehydrogenases into solution, whereas β-hydroxybutyric dehydrogenase remained bound to the particle residue.

6. Activation is interpreted as being due to an increased permeability of the mitochondrial membrane. It is suggested that two types of activation may be distinguished, depending on whether the increase is sufficiently large to allow enzyme molecules to leak out, or only to allow substrate molecules to penetrate. The former kind is irreversible whereas the latter can possibly be reversed.

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Tissue Fractionation Studies

15. INTRACELLULAR DISTRIBUTION AND PROPERTIES OF β-*N*-ACETYLGLUCOSAMINIDASE AND β-GALACTOSIDASE IN RAT LIVER

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Investigations of the last few years have disclosed that mammalian tissues contain other β-glycosidases besides the well-known and widely studied β-glucuronidase. According to a recent survey by Conchie, Findlay & Levvy (1959), the most active of these is β-*N*-acetylglucosaminidase, followed by β-galactosidase. The intracellular distribution of these enzymes has not yet been studied in detail, but Pugh, Leaback & Walker (1957) have published some preliminary observations indicating that the β-*N*-acetylglucosaminidase of rat kidney is partly associated with subcellular particles and can be released from them by treatment in a Waring Blendor.

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On the other hand, it is known since the investigations of Walker (1952) that liver β-glucuronidase is largely attached to cytoplasmic particles, where it exhibits phenomena of structure-linked latency and activation similar to those observed in this Laboratory for acid phosphatase (Berthet & de Duve, 1951; Berthet, Berthet, Appelmans & de Duve, 1951). Subsequent studies have shown that, except for the existence of a special microsomal component of β-glucuronidase, the two enzymes are closely related and belong, together with several other acid hydrolases, to a distinct group of cytoplasmic particles which have been called lysosomes (for a review, see de Duve, 1959).

In view of these facts, it seemed of interest to investigate the other β-glycosidases of rat liver with the techniques previously worked out for the study of the lysosomal hydrolases. The present paper describes the results obtained in these studies. Some of them have been briefly reported before (Sellinger & Doyen, 1959).

EXPERIMENTAL

Tissue fractionations

The tissue fractionations were performed according to de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). The mitochondrial fractions used for the activation experiments were isolated as described by Appelmans & de Duve (1955); they corresponded essentially to a combination of fractions M and L (see Table 1).

Enzyme assays

Reference enzymes. Cytochrome oxidase was assayed according to Appelmans, Wattiaux & de Duve (1955), but at 25°, glucose 6-phosphatase by the technique of de Duve *et al.* (1955). Total acid phosphatase was measured by the method of Wattiaux & de Duve (1956) in the presence of 0·1% of Triton X-100 as releasing agent or, alternatively, in the presence of 0·025% of digitonin, which, as shown in the present paper, also liberates the enzyme completely; free acid phosphatase was assayed as described by Gianetto & de Duve (1955). Nitrogen was determined by a micro-Kjeldahl procedure.

β-N-Acetylglucosaminidase. In the distribution experiments, total β-*N*-acetylglucosaminidase activity was measured according to a method derived from that of Conchie (1954) for β-glucosidase. The enzyme preparation was incubated for 30 min. at 37° in a total volume of 2 ml. containing 0·05 M-sodium citrate–HCl buffer, pH 5·0, 0·1% of Triton X-100 or 0·025% of digitonin, and 4 mM-*o*-nitrophenyl-*N*-acetyl-β-D-glucosaminide. The reaction was stopped by the addition of 3 ml. of 2·75% (w/v) trichloroacetic acid and the mixture was filtered. To 2 ml. of the filtrate were added 0·75 ml. of 0·5 N-NaOH and 1·25 ml. of 0·25 M-glycine–Na_2CO_3 buffer, pH 10·0. The *o*-nitrophenol content of this mixture was measured colorimetrically in a Klett–Summerson photoelectric colorimeter, with a violet light filter (no. 42). Tissue and substrate blanks were run concurrently; they were always very low. It was verified that under these conditions the measured activities were proportional both to enzyme concentration and to incubation time.

In the activation experiments, the free activities of β-*N*-acetylglucosaminidase and of acid phosphatase were measured simultaneously by incubating the enzyme preparation for 10 min. at 37° in a total volume of 2 ml. containing 0·25 M-sucrose, 0·05 M-pyridine–HCl buffer, pH 5·0, 0·05 M-sodium β-glycerophosphate, pH 5·0 and 4 mM-*o*-nitrophenyl-*N*-acetyl-β-D-glucosaminide. The reaction was stopped by the addition of 4 ml. of 2·75% (w/v) trichloroacetic acid. Inorganic phosphate and *o*-nitrophenol were determined on separate portions of the filtrate. Total activities were measured similarly in the presence of an emulsifying agent (usually 0·025% of digitonin). Control experiments showed that the activities measured by this procedure were identical with those determined separately by the methods described above. The pyridine buffer was chosen because it was found to inhibit neither of the two enzymes, whereas the aminidase was inhibited by acetate and the phosphatase by citrate.

β-Galactosidase. Free and total β-galactosidase activities were assayed similarly by incubating the enzyme preparation at 37° in a total volume of 2 ml. containing 0·25 M-sucrose, 0·05 M-pyridine–HCl buffer, pH 5·0, and 2·5 mM-*o*-nitrophenyl-β-D-galactoside. The amount of *o*-nitrophenol liberated was measured as in the assay of β-*N*-acetylglucosaminidase. When total activities were measured, the medium contained in addition 0·1% of Triton X-100 and the incubation time was 2 hr. The incubation time in the free activity assays was 10 min.

Materials

o-Nitrophenyl-*N*-acetyl-β-D-glucosaminide was prepared by the method of Leaback & Walker (1957), the terminal deacetylation being carried out according to Westphal & Schmidt (1952). *o*-Nitrophenyl β-D-galactoside was purchased from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). Digitonin was a Merck product and the other materials used were those mentioned in previous papers of this series.

RESULTS

Intracellular distribution

In Table 1 are shown the results of the fractionation experiments. With respect to the reference enzymes, cytochrome oxidase, acid phosphatase and glucose 6-phosphatase, the results obtained are very similar to those of de Duve *et al.* (1955), except that the nuclear fraction was more heavily contaminated with cytoplasmic material. The distribution of the glycosidases paralleled that of acid phosphatase, without, however, being entirely identical with it.

These results are represented graphically in Fig. 1. The acid phosphatase data obtained in the experiments on each glycosidase are shown separately in this graph, in order to illustrate better the correlation between the hydrolases. Slight differences in the manner in which the decantations were performed are responsible for the difference in the specific activities of the hydrolases in the two groups of experiments.

Table 1. *Intracellular distribution of enzymes*

Absolute values are given in mg./g. for nitrogen; in units*/g. (fresh wt.) of liver for enzymes. Statistics refer to means ± S.D. These are given, rather than S.E.M., since they are a measure of individual variability, the most interesting information in the present case. E, Cytoplasmic extract; N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, final supernatant.

Enzyme	No. of expt.	Absolute values E + N	Percentage values					Recovery
			E + N	N	M	L	P	S	
Nitrogen	5	31·5 ± 5·6	100	20·8 ± 5·0	17·4 ± 7·2	7·4 ± 4·0	19·8 ± 2·9	37·1 ± 9·2	102·5 ± 5·9
Cytochrome oxidase	6	27·9 ± 7·8	100	13·5 ± 5·7	55·2 ± 12·4	20·5 ± 13·4	3·4 ± 0·9	—	92·6 ± 8·4
Acid phosphatase	6	4·88 ± 0·8	100	9·9 ± 4·1	20·8 ± 3·7	37·1 ± 5·1	24·4 ± 3·0	11·9 ± 3·7	104·1 ± 5·7
β-*N*-Acetylglucosaminidase	4	6·90 ± 1·1	100	12·4 ± 4·6	33·4 ± 16·4	39·4 ± 6·9	12·4 ± 1·5	5·8 ± 0·9	103·4 ± 5·4
β-Galactosidase	2	0·34 ± 0·08	100	7·6 ± 5·6	29·4 ± 0·2	30·8 ± 1·4	10·9 ± 1·1	10·8 ± 3·0	89·5 ± 2·1
Glucose 6-phosphatase	6	14·5 ± 2·6	100	15·2 ± 6·5	3·9 ± 1·5	6·0 ± 1·6	68·2 ± 7·6	3·7 ± 3·1	97·0 ± 6·1

* Units are those defined by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955).

Fig. 1. Distribution patterns of enzymes. Fractions are plotted in the order of their isolation, i.e. (from left to right) N, M, L, P and S (see Table 1). Each fraction is represented separately in the ordinate scale by its own relative specific activity. In the abscissa scale, each fraction is represented (cumulatively from left to right) by its percentage of nitrogen (Table 1).

Activation and release

As preliminary experiments had shown that the two glycosidases are largely masked in fresh particulate fractions incubated under the conditions of the free activity measurements, a comparative investigation of the release of these enzymes and of acid phosphatase was undertaken in the line of previous studies of a similar kind (Gianetto & de Duve, 1955; de Duve *et al.* 1955; Wattiaux & de Duve, 1956).

Comparison of β-N-acetylglucosaminidase with acid phosphatase

In Fig. 2 are shown the results of an experiment in which the free enzymic activities were measured in the presence of increasing concentrations of digitonin. The release of both enzymes occurred in an essentially identical manner, and was complete in the presence of 0·01 % of digitonin. This experiment justifies the use of digitonin as releasing agent in the determination of total activities and shows that it is several times more effective in this respect than Triton X-100 (Wattiaux & de Duve, 1956). Digitonin also has the advantage that it does not interfere with the determination of inorganic phosphate as does Triton X-100. Unfortunately, it has a very low solubility in water and occasionally fails to effect complete activation owing to precipitation.

As shown in Fig. 3, repeated freezing and thawing also caused a parallel liberation of the two

enzymes. In this experiment, as in those of Bendall & de Duve (1960), freezing and thawing proved much more effective on the release of acid phosphatase than had been previously found (Gianetto & de Duve, 1955). This is presumably due to the fact that freezing was performed at a lower temperature (−80° compared with −15° in the earlier experiments).

Through the courtesy of Measuring and Scientific Instruments Ltd. we had an opportunity to submit a particulate preparation to ultrasonic vibrations. The results of this experiment, shown in Fig. 4, indicate that acid phosphatase and β-*N*-acetylglucosaminidase were both liberated by this treatment and that the phenomenon followed approximately the same course for both enzymes.

As shown in Figs. 5 and 6, β-*N*-acetylglucosaminidase was also released, as was acid phosphatase, in particulate preparations exposed to media of low osmotic pressure or pre-incubated at pH 5·0 and 37°.

The results obtained in the experiments described above and in a few others of the same type are summarized in Fig. 7, which illustrates the highly significant correlation existing between the free activities of the two enzymes in preparations subjected to graded damage. Fig. 7 also shows that the free β-*N*-acetylglucosaminidase activity of fresh untreated preparations tends to be lower than their free acid phosphatase activity.

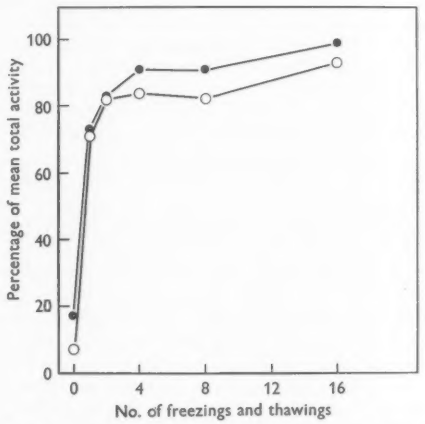

Fig. 3. Release of β-*N*-acetylglucosaminidase and of acid phosphatase by repeated freezing and thawing. Free and total activities were measured on 0·2 ml. samples of a suspension of particles in 0·25 M-sucrose (0·5 g. of original liver/ml.) frozen by dipping for 2 min. into propan-2-ol–solid carbon dioxide and then transferred first to ice–water and subsequently to a water bath at 37° until thawing was just completed. Free activities are expressed as percentages of mean total activity. ●, Acid phosphatase; ○, β-*N*-acetylglucosaminidase.

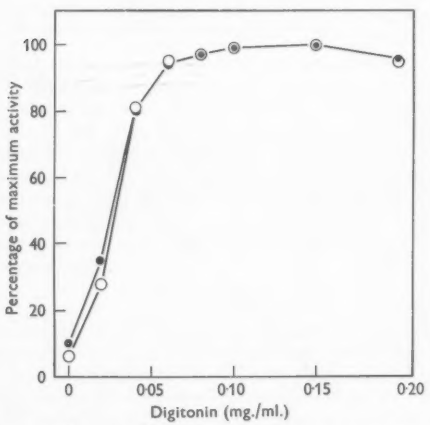

Fig. 2. Release of β-*N*-acetylglucosaminidase and of acid phosphatase by increasing concentrations of digitonin. Free activities were measured in the presence of digitonin at the concentration indicated, with an amount of particles corresponding to 0·05 g. of original liver/ml. Results are expressed as percentages of highest observed activity. ●, Acid phosphatase; ○, β-*N*-acetylglucosaminidase.

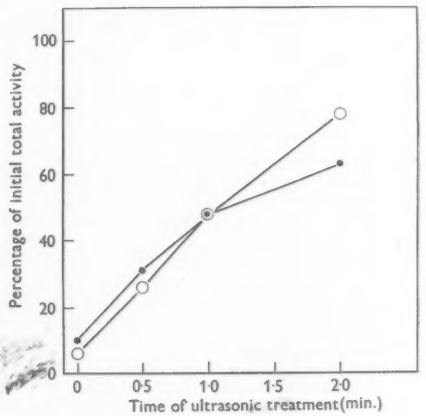

Fig. 4. Release of β-*N*-acetylglucosaminidase and of acid phosphatase by ultrasonic treatment. Free activities were measured on 0·2 ml. samples of a suspension of particles in 0·25 M-sucrose (0·5 g. of original liver/ml.) subjected at 0° and for time indicated to 20 kcyc./sec. in a MSE–Mullard ultrasonic disintegrator (60 w). Results are expressed as percentages of total activity before treatment. ●, Acid phosphatase; ○, β-*N*-acetylglucosaminidase.

A few β-*N*-acetylglucosaminidase assays were also carried out on homogenates and high-speed supernatants from liver lobes subjected to ischaemic necrosis (de Duve & Beaufay, 1959). In these experiments, which were designed to study the reversibility of the biochemical lesions induced by ischaemia, ligatures were placed on the left liver lobe and later removed after 2, 4 and 6 hr. respectively. The animals were killed 15 hr. later and the damaged and undamaged parts of the liver were homogenized and treated as described by de Duve & Beaufay (1959). The results obtained are listed in Table 2. They show that removal of the ligature after 2 hr. or more did not prevent the inactivation of cytochrome oxidase and glucose 6-phosphatase nor the release of acid phosphatase from proceeding almost to completion, but caused an appreciable loss of the latter enzyme, which did not occur when the ligature was maintained until the time of killing (de Duve & Beaufay, 1959). β-*N*-Acetylglucosaminidase was also released to a fairly considerable extent, but, unlike that of acid phosphatase, its total activity did not decrease significantly.

β-Galactosidase. The release of this glycosidase was studied according to an abridged experimental scheme allowing several treatments to be investigated simultaneously on the same preparation. Conditions were chosen so as to provide significant points on each activation curve. Free and total activities of β-galactosidase and of acid phosphatase were measured in all cases, but on separate samples, since the disparity in the activities of the two enzymes made it necessary to use a larger concentration of particles for β-galactosidase than for acid phosphatase. The results obtained are shown in

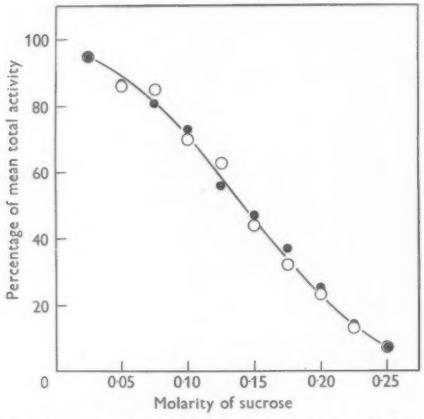

Fig. 5. Release of β-*N*-acetylglucosaminidase and of acid phosphatase by osmotic disruption. Free and total activities were measured on particles kept for 15 min. at 0° in the concentration of sucrose indicated (0·05 g. of original liver/ml.) and then brought back into 0·25 M-sucrose by a 50% dilution with a sucrose solution of suitable concentration. Results are expressed as percentages of mean total activity. ●, Acid phosphatase; ○, β-*N*-acetylglucosaminidase.

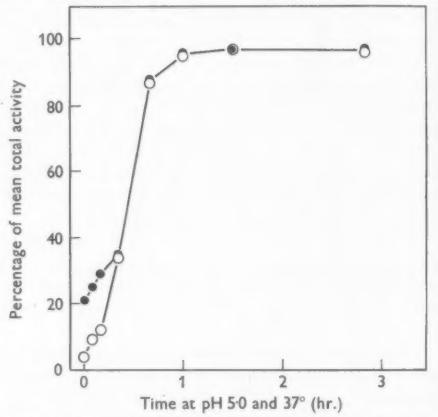

Fig. 6. Release of β-*N*-acetylglucosaminidase and of acid phosphatase by incubation at pH 5·0 and 37°. Free and total activities were measured after pre-incubation of the particles (0·1 g. of original liver/ml.) at 37° and for the time indicated in 0·25 M-sucrose containing 0·05 M-pyridine–HCl buffer. Free activities are expressed as percentages of mean total activities. ●, Acid phosphatase; ○, β-*N*-acetylglucosaminidase.

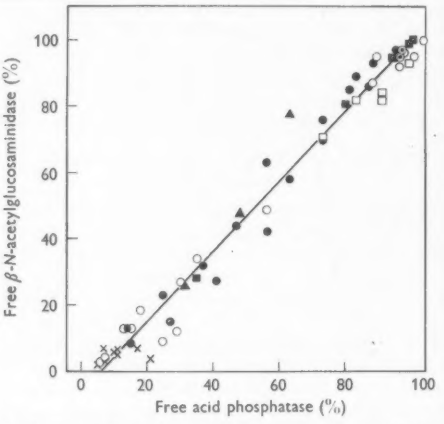

Fig. 7. Correlation between free β-*N*-acetylglucosaminidase and acid phosphatase activities of untreated particulate preparations (×) and of preparations exposed to media of lowered sucrose concentration (●), incubated at pH 5·0 and 37° (○), treated with digitonin (■), frozen and thawed (□) and exposed to ultrasonic vibrations (▲). Correlation coefficient, 0·99.

Table 2. *Influence of ischaemia on enzymic activities of liver*

The left liver lobe was ligated as described by de Duve & Beaufay (1959) and the ligature was later removed at times indicated. The animals were killed 15 hr. after removal of the ligature. The injured lobe and an undamaged part of the liver were homogenized in 0·25 M-sucrose; part of the homogenates were centrifuged for 30 min. at 100 000 *g* in the Spinco model L centrifuge with rotor no. 40. Total and free activities were measured on the homogenates, unsedimentable (unsed.) activities on the high-speed supernatants. All activities are related to wet weight of tissue. AGase, β-*N*-Acetyl-glucosaminidase.

		Percentage of total activity of unligated part				Percentage of corresponding total activity		
Expt. no.	Treatment of lobe	Cytochrome oxidase	Glucose 6-phosphatase	Total acid phosphatase	Total AGase	Free acid phosphatase	Unsed. acid phosphatase	Unsed. AGase
1	Unligated	100	100	100	100	21	5	1
	Ligated (2 hr.)	11	16	68	95	71	19	30
2	Unligated	100	100	100	100	18	6	2
	Ligated (4 hr.)	6	11	50	114	89	31	29
3	Unligated	100	100	100	100	19	7	2
	Ligated (6 hr.)	3	9	69	99	88	46	27

Table 3. *Influence of incubation medium on free β-galactosidase and acid phosphatase activities*

Fresh particulate preparation were assayed for enzymic activities as described under Methods (separate substrates), or similarly in a substrate mixture containing 0·05 M-β-glycerophosphate together with 2·5 mM-β-galactoside (combined substrates). Activities are expressed as percentages of the total activities measured with separate substrates.

	Enzymic activities			
	Acid phosphatase		β-Galactosidase	
Substrates	Total	Free	Total	Free
Separate	100	9·5	100	19·2
Combined	89·2	9·6	102·5	20·7

summarized form in Fig. 8. They indicate that the release of β-galactosidase under the influence of the applied treatments paralleled that of acid phosphatase, but that the untreated preparations had more free β-galactosidase than acid phosphatase activity. As shown by the data of Table 3, this difference cannot have been due to the difference in the assay conditions, since it was observed also when both enzymes were assayed in media of identical composition.

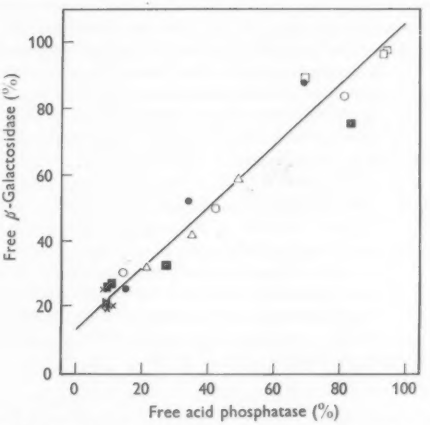

Fig. 8. Correlation between free β-galactosidase and acid phosphatase activities of untreated particulate preparations (×) and of preparations exposed to 0·175, 0·125 and 0·075 M-sucrose (●), incubated at pH 5·0 and 37° for 10, 20, 40 and 60 min. (○), exposed to digitonin concentrations of 0·025, 0·05, 0·1 and 0·25 mg./ml. at a particle concentration of 0·25 g. of original liver/ml. (■), frozen and thawed once, twice and four times (□), and treated in a MSE blender (as described by Bendall & de Duve, 1960) for 25, 60 and 180 sec. (△). Free enzymic activities were measured on separate samples of the treated suspensions and are expressed as percentages of the corresponding total activities. Correlation coefficient, 0·98.

DISCUSSION

Both by their intracellular distribution and by their structure-linked latency and mode of release under various conditions, the two glycosidases studied in the present investigation have been found to resemble acid phosphatase closely, thus duplicating a pattern of behaviour previously shown to characterize also β-glucuronidase, cathepsin and the two acid nucleases (Gianetto & de Duve, 1955; de Duve *et al.* 1955; Wattiaux & de Duve, 1956), as well as sulphatases A and B (Viala & Gianetto, 1955; Roy, 1958) and phosphoprotein phosphatase (Paigen & Griffiths, 1959). These results suggest strongly that the newly studied enzymes belong to the same group of particles as acid phosphatase and the other acid hydrolases. They also reinforce the concept, embodied in the name lysosome, that these particles are mainly concerned with acid hydrolysis.

As with the other enzymes of the lysosomal group, the distributions found for the glycosidases

are not identical with that of acid phosphatase, nor, for that matter, with that of any of the other acid hydrolases. This heterogeneity, which in the present case appears to be linked with a difference in the degree of latency of the enzymes, has been commented upon before. It seems to be a general property of lysosomes, which cannot be interpreted unequivocally at the present time (see, for instance, the discussion in de Duve, 1959). It should be noted that the glycosidases do not appear to show a second, microsomal component, as was found for β-glucuronidase, the enzyme most closely related to them functionally (de Duve *et al.* 1955).

The results described here confirm the intimate relationship between β-*N*-acetylglucosaminidase, β-galactosidase and β-glucuronidase established by Conchie *et al.* (1959). These authors, who have made observations very similar to those described in the present paper (G. A. Levvy, personal communication), have made the interesting discovery that tissues which contain the β-glycosidases also contain an α-mannosidase. According to a few preliminary experiments (performed with a sample of substrate kindly provided by Dr G. A. Levvy), α-mannosidase, another soluble acid hydrolase, appears to belong also to the lysosomes.

Conchie *et al.* (1959) have expressed the opinion that these various glycosidases may exert a concerted type of action in the catabolism of mucopolysaccharides and glycoproteins. This hypothesis is in line with the general lytic function attributed to the lysosomes. As discussed by de Duve (1959), the segregation of the hydrolases within impermeable particles probably represents a fundamental control mechanism of their lytic activity.

SUMMARY

1. The intracellular distribution of β-*N*-acetylglucosaminidase and of β-galactosidase has been investigated in rat liver according to a fractionation scheme in which the mitochondrial fraction is divided into two subfractions. It was found to differ distinctly from those of the mitochondrial cytochrome oxidase and of the microsomal glucose 6-phosphatase and to parallel closely that of the lysosomal acid phosphatase.

2. Like acid phosphatase, both enzymes occurred largely in latent form in fresh particulate preparations. Treatments which caused a release of acid phosphatase liberated the β-glycosidases in approximately the same proportion.

3. In both types of experiments, small but significant differences were observed in the behaviour of the three hydrolases.

4. It is concluded from these results that the two β-glycosidases are associated with the lysosomes and the differences observed are attributed to the heterogeneity of this class of particles, already revealed by previous investigations.

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A Micro-determination of Carbohydrates and Proteins

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Johnson (1949) described a method for estimating some proteins and carbohydrates in aqueous solution and suggested the use of the method in assessing the purity of enzymic fractions. He pointed out that biological activities quoted in terms of dry weight are less useful than those given in terms of organic material, particularly when inorganic salts are used in purification procedures. Furthermore, large samples are required to avoid errors in weighing. Dixon & Webb (1958) have also suggested the use of the method to follow enzymic purifications. Johnson's (1949) procedure uses a mixture of sodium dichromate in concentrated sulphuric acid to oxidize the sample (100–500 μg. in 0·4 ml.), after which the excess of dichromate is determined colorimetrically. The method has been applied by Halliwell (1958) to the determination of insoluble carbohydrate in the form of cellulose. Johnson's method (1949) for soluble carbohydrates and proteins is both rapid and useful, except for the amount of organic material required, particularly in enzymic work. We have developed a procedure in which only 9–40 μg. of carbohydrate or protein in up to 0·45 ml. is used. A submicro-procedure is also described for carbohydrates in amounts of 0·9–4 μg. by using one-tenth the volumes of reagents and sample. These procedures involve reaction of the excess of dichromate with starch–iodide reagent. The intensity of the blue colour is measured with a photoelectric absorptiometer. The original procedure of Johnson (1949) has also been modified and made more reliable for the range 100–700 μg. of soluble organic material by the use of more dilute dichromate–H_2SO_4 solution and by using aqueous rather than solid sulphite. It has thus been possible to obtain the theoretical calibration curves (for carbohydrates) in practice.

The new procedure is recommended for the determination of carbohydrates, proteins and their hydrolysis or breakdown products formed enzymically or otherwise, for following the purity of proteins or enzymes during fractionation procedures and for the analysis of chromatographic eluates.

MATERIALS AND METHODS

General reagents

Standard solutions of carbohydrates and proteins. Glucose (A.R.), sucrose (A.R.) cellobiose (Light and Co. Ltd.), soluble starch (A.R.), crystalline samples of pepsin, trypsin and bovine plasma albumin (Armour Laboratories), Bactopeptone and -tryptone (Difco Laboratories) were used. The products were analysed for moisture (dried at 105°) and ash content (750° for 3 hr.) and where necessary the figures quoted in the text are corrected for these values. The carbohydrates and proteins were prepared in aqueous solution at a concentration of approximately 40 μg. in 0·45 ml.

Reagents for the determination of 9–40 μg. *of organic material in* 0·45 *ml. and for* 0·9–4 μg. *in* 0·045 *ml.*

Aqueous 3·4% *(w/v) potassium dichromate.* Potassium dichromate (A.R.) (1·7 g.) is dissolved in water with heating and the solution made up to 50 ml. This solution is stable for at least 6 months at room temperature in an all-glass vessel.

Solution (0·034%, *w/v) of potassium dichromate in* 97% *(w/v) sulphuric acid.* The 3·4% (w/v) solution (0·5 ml.) of $K_2Cr_2O_7$ in water is cooled in a vessel having a ground-glass neck and 49·5 ml. (50 ml. measuring cylinder) of 98% H_2SO_4 (Micro Analytical Reagent, British Drug Houses Ltd.) is added and mixed with further cooling. This reagent is prepared just before use.

Cadmium iodide–starch reagent. Cadmium iodide (A.R.) (1·1 g.) is dissolved in 34 ml. of water and the solution boiled gently (to remove possible traces of free I_2) for 15 min., the volume being kept constant. Meanwhile starch solution is prepared by boiling 0·26 g. of soluble starch (A.R.) in 46 ml. of water for about 5 min. until the liquid clears. This solution is then added slowly to the gently boiling CdI_2 solution and the mixture boiled for about 5 min. It is then filtered through a no. 3 sintered-glass filter (or no. 50 Whatman filter paper), cooled to room temperature and made up to 100 ml. with water. The reagent is stable for at least a month (see below) in clear glass-stoppered Pyrex flasks at room temperature out of direct sunlight.

Reagents for the determination of 100–700 μg. *of organic material in* 0·9 *ml.*

Solution (0·25%, *w/v) of potassium dichromate in* 96% *(w/v) sulphuric acid.* Potassium dichromate (A.R.) (2·5 g.) is dissolved with heating in 20 ml. of water, and the solution cooled and diluted to 1 l. with 98% H_2SO_4. The reagent is stored in a glass-stoppered vessel protected from dust and is stable at room temperature for at least 3 months.

Aqueous 20% *(w/v) sodium sulphite.* A solution of 4 g. of $Na_2SO_3,7H_2O$ (A.R.) in 20 ml. of water is prepared and stored as described previously (Halliwell, 1958). It is then stable for at least 2 weeks. The stability is readily checked by mixing 0·1 ml. of 20% (w/v) Na_2SO_3 solution with 2 ml. of 0·25% (w/v) solution of $K_2Cr_2O_7$ in 96% H_2SO_4 (see above) and 6 ml. of water. The colour produced is compared in an absorptiometer (Ilford violet filters no. 601, peak transmission 430 mμ) with that of a similar solution prepared in the absence of sulphite (see 'Analytical Procedure').

Analytical procedure

Colour measurement: preparation of calibration curves for 9–41 μg. of standard solutions of carbohydrates in up to 0·45 ml. of solution by using the Spekker absorptiometer (Hilger). Suitable volumes to cover the range 0 (blank tube)—41 μg. of each of the carbohydrates and proteins (including peptone and tryptone) are pipetted into tubes and all volumes made up to 0·45 ml. with water. The blank tube is required for $K_2Cr_2O_7$ in the absence of organic material. A 0·034 % (w/v) solution (1 ml.) of $K_2Cr_2O_7$ in 97 % H_2SO_4 is added and the reactants are mixed carefully. The tubes are closed with pear bulbs and heated in a boiling-water bath for 7 min. (carbohydrates) or 20 min. (proteins) and then cooled in cold water. Water (10 ml.) is added to each tube, the contents are mixed and 0·5 ml. is removed into 8·5 ml. of 0·21 N-H_2SO_4 in Quickfit test tubes, followed by the addition of 1 ml. of CdI_2–starch reagent. The tubes are closed with glass stoppers and mixed by inversion so that the solution touches all the internal surface of the tube. The tubes are left at room temperature (19–25°) out of direct sunlight (complete darkness is not required) for 20 min. for development of the blue colour. The solutions are read against water in absorptiometer cells with a 1 cm. light-path by using Ilford orange filters (no. 607, peak transmission 600 mμ). A reagent blank (without $K_2Cr_2O_7$), which does not need boiling, is prepared by adding 0·5 ml. of 8·6 % H_2SO_4 to 8·5 ml. of 0·21 N-H_2SO_4, followed by 1 ml. of CdI_2–starch reagent. [The 8·6 % H_2SO_4 is prepared by diluting 1 ml. of 98 % H_2SO_4 with 10·45 ml. of water to correspond with the acid concentration in the 0·034 % solution of $K_2Cr_2O_7$ in 97 % H_2SO_4 after dilution of the latter with the sample volume (0·45 ml.) and 10 ml. of water.]

A 2 ml. pipette may be used to deliver the 1 ml. of the 0·034 % solution of $K_2Cr_2O_7$ in 97 % H_2SO_4 and should have a drawn-out tip with an orifice about 1 mm. in diameter. In all stages before boiling the samples with $K_2Cr_2O_7$–H_2SO_4, glass paper (Whatman) is used to wipe off excess of sample and $K_2Cr_2O_7$–H_2SO_4 from the tips of pipettes.

Submicro-determination of 0·9–4 μg. of carbohydrates in 0·045 ml. The procedure is essentially the same as that described above except that one-tenth of the volumes of reagents are used. After heating and cooling, the tubes are diluted with 1 ml. of water and then washed out by decantation with a total of 20 ml. of 0·21 N-H_2SO_4 into larger tubes: 2·3 ml. of CdI_2–starch reagent is added, the solutions are mixed and the colour is developed for 20 min. and read as described above.

Determination of 100–700 μg. of carbohydrates and proteins. This procedure follows that of Johnson (1949), with slight modifications of reagents and with further application to carbohydrates. Briefly, suitable volumes of carbohydrates and proteins containing 0 (two blank tubes are required)–700 μg. of material in 0·9 ml. of solution in Quickfit test tubes are treated with 2 ml. of a 0·25 % solution of $K_2Cr_2O_7$ in 96 % H_2SO_4 and heated in a boiling-water bath for 20 min. After cooling, and addition of 6 ml. of water and mixing, 0·1 ml. of aqueous 20 % (w/v) $Na_2SO_3,7H_2O$ is used to reduce the $K_2Cr_2O_7$ in one of the two blanks. All tubes are then read against the reduced blank with violet filters (Ilford no. 601) as above.

RESULTS

Quantitative reaction of potassium dichromate in aqueous solution and in 97 % H_2SO_4 with the cadmium iodide–starch reagent. Before the determination of organic compounds by measuring an excess of unchanged dichromate was attempted it was first necessary to show the quantitative nature of the reaction of $K_2Cr_2O_7$ with starch–iodide reagent. The stock 3·43 % (w/v) aqueous $K_2Cr_2O_7$ was diluted to 0·003 % (w/v) with water. Portions (0–0·5 ml.) were then diluted to 9 ml. by addition of 2·2 ml. of 1·63 N-H_2SO_4 and water. Cadmium iodide–starch reagent (1 ml.) was added and the blue colours were developed for 20 min. and read as described under 'Analytical Procedure'. Fig. 1 illustrates the type of curve obtained. The final concentration of $K_2Cr_2O_7$ covered the range 0·000015–0·00015 % (w/v). The final concentration of H_2SO_4 in the 10 ml. volume was about 0·36 N and gave a pH of 0·76. This concentration of acid was chosen after preliminary experiments with a range of acid concentrations had shown that the resultant pH was the most suitable for development of the blue colour. At the same time it was shown that the colour

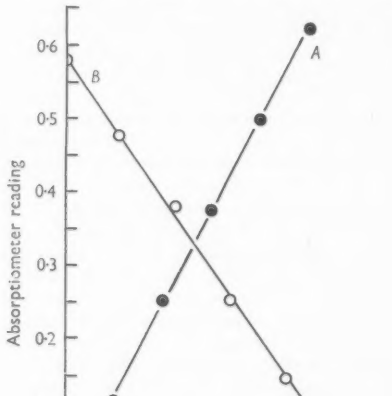

Fig. 1. *A.* Calibration curve for the reaction of $K_2Cr_2O_7$ with CdI_2–starch reagent. Aqueous $K_2Cr_2O_7$ (0–0·5 ml. of 0·003 %, w/v) was added to 2·2 ml. of 1·63 N-H_2SO_4 and water in a final volume of 9 ml., followed by 1 ml. of CdI_2–starch reagent. Colours were read as described in the text. *B.* Standard calibration curve for the determination of excess of $K_2Cr_2O_7$ by the iodometric micro-procedure after oxidation of organic material (glucose 9–41 μg., in this case) by 1 ml. of a 0·034 % solution of $K_2Cr_2O_7$ in 97 % H_2SO_4.

developed as well at 25° as at 16 ± 3°. All colours were read against water about 20 min. after mixing the $K_2Cr_2O_7$ solutions with CdI_2–starch reagent. A reagent blank containing all solutions except $K_2Cr_2O_7$ was included. All readings increased slightly during the next 30 min. (total colour development time of 50 min.), the increase on the maximum reading being about 3 %. Readings then remained constant for a further 25 min. (total colour development time of 75 min.) with no sign of precipitation.

Having shown the quantitative response of aqueous $K_2Cr_2O_7$ with starch–iodide reagent we then attempted a similar demonstration with a 0·034 % solution of $K_2Cr_2O_7$ (w/v) in 97 % H_2SO_4 the reagent used for oxidation of carbohydrates and proteins. The acid dichromate was diluted with 10·45 vol. of water to give 0·003 % (w/v) $K_2Cr_2O_7$ in acid. Portions (0–0·5 ml.) were added to 8·5 ml. of 0·21 N-H_2SO_4 and sufficient 8·6 % H_2SO_4 was added to give a total volume of 9 ml. (the 8·6 % H_2SO_4 was prepared by diluting 1 vol. of 98 % H_2SO_4 with 10·45 vol. of water). Cadmium iodide–starch reagent (1 ml.) was added and the blue colours were developed for 20 min. and read as described under 'Analytical Procedure'. A line similar to that of Fig. 1*A* was obtained, thus indicating that the linear response also applied to $K_2Cr_2O_7$ in conc. H_2SO_4 after suitable dilution. Thus it was now possible to examine the oxidation of micro-quantities of organic compounds with dichromate in conc. H_2SO_4 followed by iodometric determination of the excess of $K_2Cr_2O_7$. The method is described under 'Analytical Procedure' (see also Fig. 1*B*).

Table 1. *Amounts of organic material found equivalent to potassium dichromate in sulphuric acid (iodometric micro-procedure)*

Dichromate equivalents are the amounts (μg.) of organic material oxidized by 1 ml. of a 0·034 % (w/v) solution of $K_2Cr_2O_7$ in 97 % (w/v) H_2SO_4, as determined by the present iodometric micro-procedure with calibration curves prepared for each compound. The theoretical equivalent for the standardized $K_2Cr_2O_7$ in H_2SO_4 is 50 μg. of glucose. Figures in parentheses under 'dichromate equivalents' are arbitrary values with glucose set at 100 (see text).

Organic material	Dichromate equiv. (mean value, μg.)	Range of values obtained for dichromate equiv. (%)
Albumin	60	99–100
Trypsin	63	98–101
Pepsin	59	98–101
Tryptone	61	98–102
Peptone	62	99–101
Cellobiose	46 (94)	98–102
Starch	44 (90)	98–101
Glucose	49 (100)	98–101

Table 2. *Amounts of organic material found equivalent to potassium dichromate in sulphuric acid (semimicro-procedure)*

Dichromate equivalents are the amounts (μg.) of organic material oxidized by 2 ml. of a 0·25 % (w/v) solution of $K_2Cr_2O_7$ in 96 % (w/v) H_2SO_4 determined by the semimicro-procedure of Johnson (1949) as modified under 'Analytical Procedure'. Equivalents are calculated from calibration curves prepared for each compound. Figures in parentheses under 'dichromate equivalents' are arbitrary values with glucose set at 100 (see text).

Organic compound	Dichromate equiv. (mean value, μg.)	Range of values obtained for dichromate equiv. (%)	Theoretical value for dichromate equiv. (μg.)
Cellobiose	725 (95)	100–101	724
Starch	687 (90)	100–101	688
Glucose	764 (100)	99–101	764

Determination of carbohydrates and proteins. Table 1 shows the amount of each compound equivalent to 1 ml. of the 0·034 % solution of $K_2Cr_2O_7$ in 97 % H_2SO_4 used in the oxidation. The equivalents were obtained by extrapolating to zero the calibration curves prepared for each compound in a manner similar to that for glucose of Fig. 1*B*. The method measures excess of $K_2Cr_2O_7$ present after oxidation of the organic compounds and the curve so obtained is in effect a 'colorimetric back titration'. Determination of glucose and starch in amounts of 0·9–4 μg. by the submicro-procedure (see 'Analytical Procedure') gave similar curves. Determination of proteins in such small amounts (0·9–4 μg.) was less successful, possibly owing to the longer heating period of 20 min.

Carbohydrates were also determined by the semimicro-procedure with 100–700 μg. of organic material in 0·9 ml. Equivalents were determined in a manner similar to that described above and are given in Table 2.

The theoretical reaction for oxidation of a monosaccharide, disaccharide and polysaccharide (we have used glucose, cellobiose and starch) may be represented thus:

$$\underset{180}{C_6H_{12}O_6} \text{ or } \tfrac{1}{2}\underset{171}{C_{12}H_{22}O_{11}} \text{ or } \underset{162}{C_6H_{10}O_5} + 6O_2 \rightarrow 6CO_2 + \begin{cases} 6H_2O \\ 5\tfrac{1}{2}H_2O \\ 5H_2O \end{cases}$$

from which it can be calculated that 1·177 g. of $K_2Cr_2O_7$ oxidize 180 mg. of glucose, 171 mg. of cellobiose or 162 mg. of starch. The ratio monosaccharide : disaccharide : polysaccharide is then 100 : 95 : 90. The theoretical equivalents in Tables 1 and 2 have been calculated from the above equation. Within experimental error the experimental values agree with the theoretical equivalents. Owing to the complex nature of the oxidation of proteins it does not appear possible to formulate simple theoretical equivalents, although the experimental values occur in a narrow range.

Recovery of cellobiose, sucrose and starch by using a glucose calibration curve. The carbohydrates were estimated by the iodometric micro-procedure in amounts of 10 and 40 μg. in 0·45 ml. of solution. Recoveries were calculated from a standard glucose curve by using the factors 0·95 and 0·90 to convert the 'glucose' values of the determined disaccharides and polysaccharide into anhydroglucose values as described above. Table 3 indicates the recoveries obtained.

Stability of dilute potassium dichromate in 97 % sulphuric acid. This reagent, prepared as under 'Analytical Procedure', was made up at three concentrations of dichromate at approximately 0·034 % (w/v) (the concentration used in the present iodometric procedure), 0·017 and 0·003 % (w/v) in 97 % H_2SO_4. Samples of each solution were heated in a boiling-water bath for periods up to 2 hr., cooled and compared with a corresponding sample of unheated solution of $K_2Cr_2O_7$, in 97 % H_2SO_4. Comparisons were made by the present iodometric micro-method on the absorptiometer. A sample of a 0·034 % (w/v) $K_2Cr_2O_7$ in 97 % H_2SO_4 was also examined for its stability at room temperature.

The 0·034 % (w/v) solution in 97 % H_2SO_4 represents the minimum concentration of $K_2Cr_2O_7$ in 97 % H_2SO_4 suitable for general use in the oxidation of organic material. Smaller concentrations (0·017 %, w/v) of $K_2Cr_2O_7$ in 97 % H_2SO_4 are partly destroyed on boiling or completely destroyed [0·003 % (w/v) $K_2Cr_2O_7$]. Even at room temperature (up to 20°) the 0·034 % solution in 97 % sulphuric acid loses some potency on storage for a few days. As a result it was found more convenient to prepare the 0·034 % (w/v) solution of $K_2Cr_2O_7$ in 97 % H_2SO_4 (the reagent used for oxidation of carbohydrates and proteins), when needed, by adding approx. 99 vol. of 98 % H_2SO_4 to 1 vol. of 3·4 % (w/v) aqueous $K_2Cr_2O_7$ (see 'Analytical Procedure'). The 3·4 % (w/v) aqueous $K_2Cr_2O_7$ is stable for at least six months in an all-glass vessel at room temperature.

Cadmium iodide–starch reagent. In order to estimate micro-quantities of organic material it is necessary to use very dilute solutions of $K_2Cr_2O_7$ for the oxidation. Hence a sensitive method is required for determination of the excess of $K_2Cr_2O_7$. The use of unstable KI and starch solutions was avoided by employing one solution, the CdI_2–starch reagent of Lambert (1951). This reagent is colourless, highly sensitive and stable for at least a month when prepared with soluble starch as described under 'Analytical Procedure'. Lambert & Rhoads (1956) found that the reagent was even more stable (for 6 months) when pure linear starch fraction ('amylose') was used. They prepared this fraction by a method which uses a combination of the procedures of Krishnaswamy & Sreenivasan (1948)

Table 3. *Recovery of cellobiose, sucrose and starch as measured by the iodometric micro-procedure*

Approximately 10–40 μg. of the carbohydrates was estimated by the iodometric micro-procedure and the results were calculated as anhydroglucose from a standard glucose calibration curve.

Carbohydrate	Amount taken in 0·45 ml. of soln. (μg.)	Amount found (mean value, %)	Range of recovery (%)
Cellobiose	41	101	99–103
	8·6	99	97–101
Sucrose	41	101	101–102
	8·9	97	96–100
Starch	32	98	97–100
	9·1	96	95– 99

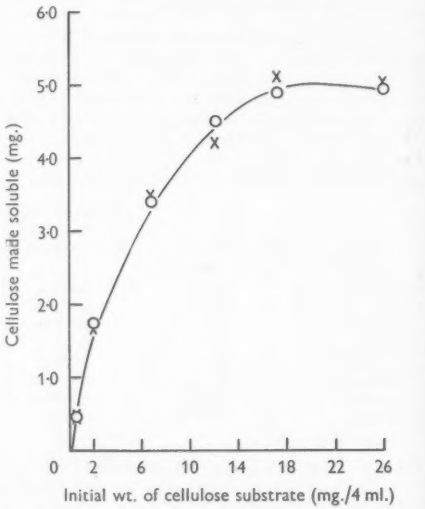

Fig. 2. Effect of substrate 'concentration' on the breakdown of insoluble cellulose to soluble products by enzyme preparations from *Myrothecium verrucaria*. A volume (0·5 ml.) of standard enzyme preparation in Sörensen phosphate buffer (pH 5·5, 0·067 M-Na_2HPO_4 + 0·067 M-KH_2PO_4) was incubated at 37° for 1 hr. with the substrate, swollen cellulose powder (Whatman) (Halliwell, 1957). ×, Loss of weight of insoluble cellulose determined from the residual weight of cellulose after incubation with enzyme, followed by filtration and washing of cellulose (Halliwell, 1958); ○, soluble saccharides produced (estimated as glucose, expressed as cellulose). The soluble saccharides were separated by filtration and determined by iodometric estimation of excess of $K_2Cr_2O_7$. The protein of the enzyme used gave a negligible dichromate value in comparison with that of the soluble saccharides formed.

and of Schoch (1945). We have obtained the linear starch fraction by the same methods and used it to prepare the CdI_2–starch reagent when it gave readings 5–10% greater than did soluble starch. Use of the purified starch, however, was discontinued when it was found that CdI_2–starch reagent prepared with commercially-available soluble starch was stable for at least a month. The stability of the reagent was examined by adding 1 ml. of the solution to 0·5 ml. of 0·003% (w/v) aqueous $K_2Cr_2O_7$ in 2·2 ml. of 1·63N-H_2SO_4 in a total volume of 10 ml. with water. The blue colour was developed for 20 min. and read as described above.

Application of the method to the determination of cellulose breakdown by enzymic preparations. The iodometric micro-procedure has been used to determine the formation of soluble carbohydrates formed from different amounts of cellulose by enzymes in a culture filtrate from *Myrothecium verrucaria* (Fig. 2).

The soluble products were separated by filtration through sintered glass (porosity 3) with or without prior centrifuging. Centrifuging by itself is insufficient to precipitate the finer particles of cellulose which remain floating on the supernatant fluid, a difficulty encountered previously (Halliwell, 1958). In addition to determining soluble products we also determined residual cellulose as described earlier (Halliwell, 1958) in order to obtain a direct estimate of the amount of insoluble cellulose which had been made soluble. The experiment in Fig. 2 was designed to study the effect of the 'concentration' of insoluble cellulose on the amount of insoluble cellulose made soluble by a fixed amount of enzyme. There is reasonable agreement between the results obtained by the two methods used for the determination of cellulolysis. The enzyme appears to be saturated with insoluble cellulose at about 20 mg.

DISCUSSION

The main errors of the iodometric micro-method were found to be associated with delivery of the dilute solution of $K_2Cr_2O_7$ in 97% H_2SO_4. This was largely overcome by the use of a pipette with a drawn-out tip similar to that of a Warburg pipette (Gallenkamp, cat. no. a 12229). If necessary the tip of the pipette may be ground down to give an orifice sufficiently wide (approximately 1 mm.) to allow a reasonable rate of outflow of the $K_2Cr_2O_7$–H_2SO_4 mixture. The pipette tip is immersed in the reagent to the minimum depth necessary for filling, and excess of solution is wiped off with glass-fibre papers (Whatman).

The reaction of $K_2Cr_2O_7$–H_2SO_4 is rapid when carbohydrates are oxidized (7 min. heating period) and temperatures between 95° and 100° are equally satisfactory. Proteins require 20 min. heating and it is important to maintain the water bath at boiling point.

We have used the iodometric-dichromate procedure to determine the total soluble material produced by enzymic degradation of insoluble cellulose. In order to examine true enzymic breakdown of insoluble cellulose it is necessary to determine residual insoluble cellulose or alternatively to determine the total soluble carbohydrates produced by the assay medium in the presence and absence of enzyme (Halliwell, 1959). An important feature of the present method when used to follow cellulolysis, in contrast with those methods dependent on the measurement of the formation of 'reducing' sugars, is that it enables one to measure and correct for that fraction of the substrate which consists of small molecules, whether reducing or non-reducing, that are soluble in the assay medium in the absence of enzyme. This fraction, we have found, can on occasions amount to 1–2% of the cellulose added and could presumably be attacked by β-glucanases (Festenstein, 1959) in the enzyme preparation. It is perhaps significant that most of the 'cellulases' in the literature will accomplish no more than this degree of dissolution of insoluble substrate. This fraction cannot be corrected for in the usual reducing-sugar methods, since the soluble small-chain polysaccharides have negligible reducing power. In the 'weight-loss' method for following cellulolysis (Halliwell, 1958), the soluble fraction is automatically corrected for, since the degree of cellulolysis is calculated from the substrate control, taken as 100% initial cellulose.

SUMMARY

1. An iodometric micro-method is described for the quantitative determination of carbohydrates and proteins by oxidation with a solution of potassium dichromate in concentrated sulphuric acid. The excess of reagent is determined colorimetrically by the addition of a starch–iodide solution. Glucose, cellobiose, sucrose and starch can be determined from a calibration curve for glucose.

2. The method covers the range 9–40 μg. of carbohydrates and proteins. Recovery for cellobiose at about 9 μg. is 99% (range 97–101%), and at 41 μg. 101% (range 99–103%); for soluble starch at 9 μg. recovery is 96% (range 95–99%) and at 32 μg. 98% (range 97–100%).

3. The procedure is recommended for the determination of micro-quantities of carbohydrates, proteins and their breakdown products formed enzymically or otherwise, for following purity of enzymes or proteins during fractionation procedures and for analysis of chromatographic eluates.

4. The procedure has been applied to determine the conditions which control the breakdown of

insoluble cellulose to soluble products by enzymes from *Myrothecium verrucaria*.

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The Effect of Hypophysectomy of the Rat and of Treatment with Growth Hormone on the Incorporation *in vivo* of Radioactive Amino Acids into the Proteins of Subcellular Fractions of Rat Liver

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The liver of the rat, like the body as a whole, decreases in size and protein content as a result of hypophysectomy, and this fall can be prevented to a large extent by treatment of the rat with growth hormone (Simpson, Evans & Li, 1949). There is some evidence that growth hormone stimulates growth by increasing the rate of protein biosynthesis (Russell, 1955) rather than by slowing down the rate of catabolism of proteins.

Zamecnik & Keller (1954) have described the preparation of a cell-free system from rat liver which brings about the incorporation of radioactive amino acids into protein *in vitro*. In the soluble fraction of the cell the amino acids are prepared for incorporation into proteins by amino acid-activating enzymes (Hoagland, Keller & Zamecnik, 1956) and by forming an amino acid-soluble ribonucleic acid intermediate (Hoagland, Zamecnik & Stephenson, 1957). In this system the assembly of the polypeptide chains occurs in the ribonucleoprotein microsomal particles of the endoplasmic reticulum (Littlefield, Keller, Gross & Zamecnik, 1955).

With a system of the type described by Zamecnik & Keller (1954), Korner (1958, 1959) has shown that the microsomes from the livers of hypophysectomized rats are significantly less able than those from livers of normal rats to assemble amino acids into proteins. Hypophysectomy of the rat did not result in any change, that was detectable by the system used *in vitro*, in the ability of the soluble fraction of the liver cells to prepare amino acids for incorporation. Treatment of hypophysectomized rats with growth hormone restored, to some extent, the diminished ability of the liver microsomes to incorporate amino acids into protein. Treatment of normal rats with growth hormone enhanced the ability of liver microsomes to incorporate amino acids into protein.

The experiments described in this paper were carried out to see how far these findings *in vitro* could be confirmed and extended by experiments on the incorporation of amino acids into proteins in whole animals.

METHODS

Animals. Young, adult, female albino rats, of the same age and body weight, were selected from an inbred strain and were placed, at random, into two groups. The rats of one group were hypophysectomized under ether anaesthesia by the parapharyngeal approach (Ingle & Griffith, 1942), and the rats of the second group were submitted to a sham operation. After recovery from the operation each rat was housed in an individual cage and all rats were given the same ration of powdered diet (diet 41, Bruce & Parkes, 1949) made into a thick paste with water. The daily ration, established by trial and error, was the largest quantity of food which was entirely consumed by every rat (approx. 10 g./100 g. of rat/day). Water was freely available. The animals were kept on this ration for 3 weeks before being used in an experiment. The rats were not starved during the night before they were killed although each rat had eaten its last ration several hours before it was killed.

Growth hormone. Ox growth hormone, prepared by the method of Wilhelmi, Fishman & Russell (1948), was used. It was dissolved in 0·9% NaCl which had been adjusted to pH 7·5–8·0 with 0·1 N-NaOH. The dose was injected subcutaneously in 0·5 ml. of 0·9% NaCl and the control rats were given 0·5 ml. of 0·9% NaCl by subcutaneous injection.

Radioactive compounds. Generally (G) labelled L-[G-^{14}C]-leucine, DL-[1-^{14}C]leucine and hydrolysed algal protein

labelled with ^{14}C were used and were obtained from The Radiochemical Centre, Amersham, Bucks. Their specific activities were 66·9, 40·6 and 88 μc/mg. respectively. They were dissolved in such a volume of 0·9% NaCl at pH 7·0 that the appropriate dose (between 10 and 20 μc/rat) could be administered in a volume of 0·5 ml. Every rat in an experiment was given the same amount of radioactivity and no attempt was made to correlate the dose administered to a rat with its body wt. The body weights of the rats used were between 145 and 160 g. 1 μc of [^{14}C]amino acid would record about $6{\cdot}5 \times 10^4$ counts/min. on the Geiger–Müller end-window (1·5–2·5 mg./cm.^{2}) counter used.

Experimental procedure. The rats were anaesthetized with Nembutal and the femoral vein was exposed. The dose of radioactive amino acid was injected into the vein in 0·5 ml. of 0·9% NaCl. A small incision was made in the abdominal wall above the liver and gentle pressure on the sides of the abdomen forced the first lobe of the liver into the incision. The blood vessels leading to this lobe were tied off and the lobe was removed. Two types of experiment were carried out. In one, the specific activity of the unseparated liver proteins of each lobe was determined, together with the specific activity of the free intracellular amino acids, and in the other, the liver tissue was separated into subcellular components and the specific activity of the proteins of each was determined. In the first type of experiment each lobe of liver as it was removed from the rat was plunged into boiling water, cut up with scissors and boiled. In the second type of experiment each liver lobe was placed in ice-cold 0·35 M-sucrose containing an excess of unlabelled amino acids corresponding to the radioactive ones injected. The liver was cut into small pieces with scissors. The exact time interval, from the end of the injection of the radioactive amino acids until the moment the liver was placed in the boiling water or in the cold sucrose, was noted (2–3 min.). The second lobe of the liver was removed in a similar manner 8–9 min. after the injection of the amino acids and the remainder of the liver was removed 20 min. after the injection, and each lobe was again placed in boiling water or in ice-cold sucrose, according to the experiment being carried out. Just before the last lobe of liver was removed, a sample of blood was taken by cardiac puncture with an oxalate-treated hypodermic syringe and the blood was added to boiling water. The blood proteins were precipitated and washed in the manner described below for liver tissue and the combined washings were used to determine the specific activity of the free amino acids in blood.

Determination of total protein specific activity and intracellular amino acid specific activity. The boiled liver was thoroughly homogenized in a tight-fitting, motor-driven, glass homogenizer and the brei washed with water into the centrifuge tube in which it had been boiled. An equal volume of 0·5 N-perchloric acid was added to the brei to ensure precipitation of all the protein. The precipitated protein was centrifuged at 600 *g*. The supernatant fluid was removed and the protein precipitate was resuspended by homogenization, once in water and once in 0·25 N-perchloric acid and the precipitated protein spun down each time by centrifuging. These washings were combined with the original supernatant fluid and were used to determine the specific activity of the free intracellular amino acids, and the protein was prepared for counting in the way described below.

Triplicate 0·5 ml. samples of the protein-free washings of the protein of each tissue sample were pipetted on to disks of lens paper on lacquered aluminium planchets and 0·1 ml. of a dilute polyvinyl alcohol soln. was added to ensure even spreading. The sample was dried under an infrared lamp and the radioactivity determined in a Geiger–Müller windowless, gasflow counter. Enough counts were recorded to reduce the standard error to 3% or less and a correction was applied for the background counts. Three further 0·5 ml. samples of the combined washings were used for the determination of the amino acid content of the washings by a modification of the ninhydrin method of Moore & Stein (1948). Each sample was diluted with water to 2 ml. and 0·2 ml. of a 10% solution of recrystallized ninhydrin in 2-methoxyethanol was added. A sample (1 ml.) of a freshly prepared mixture consisting of 2 ml. of 0·01 M-KCN, 23 ml. of 8·3 M-sodium acetate–1·66 M-acetic acid and 75 ml. of 2-methoxyethanol was added and the tubes were capped and boiled for 20 min. After boiling, the tubes were cooled in running water for 5 min. and 6·3 ml. of 60% ethanol was added and the mixture shaken vigorously for 30 sec.; the absorption at 570 mμ was read in the Beckman spectrophotometer. The α-amino N content (μg.) of the sample was calculated from a standard curve constructed for valine. The counts/min./μg. of α-amino N was calculated. In experiments where hydrolysed protein labelled with ^{14}C was used as the source of radioactivity, this method gives an estimate of the specific activity of the intracellular free amino acids which is of fair accuracy. There is, however, an assumption that the radioactivity measured is all present in the free amino acids. It is possible that small peptides may be present and may be radioactive. However, when samples were hydrolysed by treatment with 6 N-HCl at 100° for 24 hr. before determining the amino acid specific activity, no significant alteration in specific activity was found, compared with that of unhydrolysed samples. Radioactive nucleotides may also be present in the sample, but any differences which may exist between the specific activities of free intracellular nucleotides in differently treated rats was considered to be small enough to be neglected. Similarly, the presence in the sample of amino acids which were not present in the mixture of injected radioactive amino acids was ignored.

Separation of cell fractions. When each sample of liver had been cooled, it was removed into fresh, cold 0·35 M-sucrose and homogenized with a hand-operated homogenizer kept cold in an ice bath. Nuclei, mitochondria, microsomes and the soluble fraction of the cell were separated by differential centrifuging of the homogenate by a modification of the procedure described by Hogeboom, Schneider & Palade (1948). The tissue brei was centrifuged at 15 000 *g* for 10 min. at 0° to precipitate nuclei, mitochondria and cell debris. The supernatant fluid was carefully removed from the pellet and microsomes and the soluble fraction of the cell were separated from it in the way described below.

The pellet from the centrifuging at 15 000 *g* was resuspended by homogenization in cold 0·35 M-sucrose and the brei was centrifuged at 800 *g* for 10 min. at 0°, when the nuclei and cell debris were spun down. The supernatant fluid was carefully removed and mitochondria were separated from it (see below). The pellet containing nuclei and cell debris was suspended in cold 0·35 M-sucrose and centrifuged at 200 *g* to remove cell debris. The supernatant fluid was recentrifuged at 200 *g* and then at 800 *g* to spin down

the nuclei. The nuclei were washed twice by resuspension in 0·35 M-sucrose followed by centrifuging at 800 *g*. They were then suspended in water and their proteins precipitated with cold 0·5 N-perchloric acid containing unlabelled amino acids.

The supernatant fluid from the first centrifuging at 800 *g* was centrifuged again at 800 *g* for 10 min. The supernatant fluid was removed and centrifuged at 8000 *g* for 10 min. at 0°, when mitochondria were spun down. The supernatant fluid was discarded. The mitochondrial pellet so obtained was washed twice by resuspension in cold 0·35 M-sucrose, followed by centrifuging at 6000 *g* for 10 min. and then the mitochondria were suspended in water and their proteins precipitated with cold 0·5 N-perchloric acid containing unlabelled amino acids.

The supernatant fluid from the first 15 000 *g* centrifuging was centrifuged at 105 000 *g* for 1 hr. at 0° in a Spinco model L preparative ultracentrifuge. The supernatant fluid was removed from the microsome pellet and the proteins of the soluble fraction of the cell were precipitated with cold 0·5 N-perchloric acid containing unlabelled amino acid. The microsome pellet and the sides of the tube were carefully rinsed with ice-cold 0·35 M-sucrose and the tube was wiped with tissue paper. The microsome pellet was treated in one of two ways. Either it was suspended in water and its proteins were precipitated with cold 0·5 N-perchloric acid, or the microsome pellet was suspended by homogenization in a hand-operated homogenizer in 2 ml. of cold 0·2 M-glycylglycine buffer, pH 8·0, and treated with cold, freshly prepared 5% sodium deoxycholate soln. in 0·2 M-glycylglycine buffer, pH 8·0. The pH was determined with a glass-electrode pH meter standardized against a standard buffer soln. made in accordance with the manufacturer's instructions from Burroughs Wellcome and Co. N.B.S. buffer solution tablets. The pH of the standard soln. was 6·99 at 25°. The volume of sodium deoxycholate soln. added was that which was calculated to contain 0·75 mg. of sodium deoxycholate/mg. of microsomal protein. The suspension was kept in ice for 10 min. and a volume of ice-cold water was then added to give a deoxycholate concentration of 0·5% and the suspension was centrifuged at 105 000 *g* for 2 hr. It has been shown (Littlefield *et al.* 1955; Palade & Siekevitz, 1956) that treatment of microsome pellet in this fashion renders soluble much of the protein of the microsomes, leaving the ribonucleoprotein particles of the endoplasmic reticulum undissolved. After the centrifuging, the supernatant fluid of protein soluble in deoxycholate was carefully removed from the pellet of ribonucleoprotein microsomal particles and its proteins were precipitated with cold 0·5 N-perchloric acid containing unlabelled amino acids. The pellet of microsomal particles was suspended by homogenization in water and its proteins were precipitated with 0·5 N-perchloric acid in a similar fashion. The ribonucleic acid/protein ratio of the pellet of microsomal particles was determined and served as a guide to the degree of separation of the ribonucleoprotein particles from the rest of the endoplasmic reticulum. This ratio was between 0·65 and 0·85.

Preparation of the radioactive protein for counting. The precipitated proteins were washed, lipid and ribonucleic acid (RNA) were extracted and the protein was dried, plated and its radioactivity determined by counting in a Geiger–Müller end-window counter (1·5–2·5 mg./cm.²) in the way previously described (Korner, 1959). The results are given as counts/min./mg. of protein after corrections have been made for self-absorption and background counts.

Determination of ribonucleic acid. The RNA content of samples of the liver fractions was determined by a slight modification of the method of Scott, Fraccastoro & Taft (1956) as previously described (Korner, 1959).

Determination of protein content. The N content of samples of the cell fractions was determined by the micro-Kjeldahl technique and a correction applied for the N content of any RNA present. The corrected value for N was used to calculate the protein content of the samples by using the factor 6·25.

Statistical treatment of results. At least four rats were used for each experimental procedure and the experimental observations were used to calculate the mean value and the standard error of the mean (S.E.M.). Student's *t* test was applied to ascertain if differences between mean values were statistically significant, and values of Fisher's *P* are given where differences between means are considered to be significant.

RESULTS

Table 1 shows the mean specific activities of the unseparated liver proteins of each of the three lobes of liver of normal rats, of hypophysectomized rats and of hypophysectomized rats given 0·3 mg. of growth hormone/day for 5 days. The lobes were removed 2, 8 and 20 min. after injection of 20 μc of hydrolysed [^{14}C]protein. Hypophysectomy has caused a statistically significant reduction in the incorporation of the radioactive amino acids into protein and treatment of the hypophysectomized rats with growth hormone has resulted in more

Table 1. *Effect of hypophysectomy of the rat, and of treatment of hypophysectomized rats with 0·3 mg. of growth hormone/day for 5 days, on the incorporation of intravenously injected algal protein hydrolysate labelled with* ^{14}C (20 μc) *into the proteins of the three lobes of liver*

Lobe of liver	Time lobe removed after injection of amino acids (min.)	Specific activity of protein (mean ± S.E.M.; four rats in each group)			Fisher's *P* less than	
		Normal rats (N)	Hypophysectomized rats (H)	Growth hormone-treated hypophysectomized rats (HG)	N, H	H, HG
1	2	62 ± 5·2	41 ± 4·1	53 ± 3·2	0·01	0·05
2	8	101 ± 7·6	64 ± 5·7	86 ± 5·9	0·01	0·02
3	20	123 ± 9·3	82 ± 6·9	103 ± 6·8	0·01	0·05

Table 2. *Effect of hypophysectomy of the rat, and of treatment of hypophysectomized rats with 0·3 mg. of growth hormone/day for 5 days, on the specific activity of the free amino acids in liver and blood, after intravenous injection of hydrolysed algal protein labelled with* ^{14}C (20 μc)

Lobe of liver	Time lobe removed after injection of amino acids (min.)	Counts/min./μg. of α-amino N (mean ± S.E.M.; four rats in each group)		
		Normal rats	Hypophysectomized rats	Growth hormone-treated hypophysectomized rats
1	2	46±6·3	41±4·3	44±5·1
2	8	29±4·6	39±5·2	36±4·1
3	20	32±4·1	38±4·2	31±3·9
Blood	19	94±17·3	84±16·2	99±19·5

Table 3. *Effect of hypophysectomy of the rat, and of treatment of hypophysectomized rats with 0·4 mg. of growth hormone/day for 6 days, on the incorporation of intravenously injected* DL-[1-^{14}C]*leucine* (15 μc) *into the proteins of the nuclei, mitochondria, microsomes and soluble fraction of the three lobes of liver*

Lobe 1 was removed 2 min., lobe 2, 8 min. and lobe 3, 20 min. after injection of the amino acid.

Type of rat	Lobe of liver	Protein specific activity (mean ± S.E.M.; six rats in each group)			
		Nuclei	Mitochondria	Microsomes	Soluble fraction
Normal	1	47±4·1	49±3·8	275±17·9	30±2·6
	2	55±4·7	73±5·1	430±51·1	59±3·9
	3	77±4·9	118±9·2	312±36·2	81±6·9
Hypophysectomized	1	34±3·1	29±1·9	189±19·5	19±1·6
	2	46±3·9	55±3·2	240±27·6	33±5·0
	3	61±4·2	82±6·2	285±22·0	39±3·2
Growth hormone-treated hypophysectomized	1	39±2·9	35±3·4	235±24·5	22±2·9
	2	50±4·1	63±5·1	336±36·3	52±4·6
	3	66±4·6	99±8·3	316±36·6	68±7·1

amino acid being incorporated into liver protein than was the case with the untreated hypophysectomized rats. The increased uptake resulting from treatment with growth hormone is, at best, only probably significant statistically.

Table 2 records the specific activities of the free intracellular amino acids and it can be seen that no significant differences were found between the three groups. The scatter of the individual readings was large but it would appear that, whereas the specific activity of the intracellular free amino acids of the livers of normal rats and of hypophysectomized rats treated with growth hormone fell from an initial high level, that of the hypophysectomized rats tended to maintain a fairly constant level for the experimental period of 20 min. Little difference was apparent between the specific activities of the free amino acids of serum in the three groups of animals.

When the liver lobes were divided into the four main subcellular fractions of nuclei, mitochondria, microsomes and soluble fraction of the cell, it became clear that the specific activities of the proteins of these subcellular fractions from hypophysectomized rats were all significantly less than those of the corresponding fractions from normal rats (Table 3). The diminished incorporation of amino acids into the proteins of liver fractions from hypophysectomized rats is particularly marked in the microsomes and the soluble fraction of the cell, but it is apparent, albeit to a lesser extent, in the other two cell components. Here again, treatment of the hypophysectomized rats with growth hormone restored, to a large extent, the ability of the liver to incorporate amino acids into the proteins of each of the four cell fractions but treatment of hypophysectomized rats with growth hormone failed to cause complete restoration of the ability to incorporate amino acids into protein to the extent shown by normal rats.

When the microsome pellet was divided into microsomal particles and material soluble in deoxycholate, it was found (Fig. 1) that a very rapid rise in protein specific activity occurred in the microsomal particles of normal rats, reaching a maximum value within 2 min. The deoxycholate-soluble fraction showed a slower rise in specific activity, but the rise in specific activity continues for much longer than was the case with the microsomal particles. The specific activity of the proteins of the soluble fraction of the cell rises at the slowest rate. These results are similar to those

reported by Littlefield *et al.* (1955) except that these workers showed that the specific activity of the protein of the microsomal particles fell below that of the deoxycholate-soluble material about 12 min. after the injection of a small dose of radioactive amino acid. This 'cross over' of the specific-activity curves was not found in the experiments reported here, in which somewhat larger doses of radioactive amino acids were used (see Discussion).

These results are consistent with the idea that the ribonucleoprotein of the microsomal particles acts as a template on which peptide chains are formed from acyl-activated amino acids. Once formed, the peptide chain peels off the template and is passed into that part of the microsome soluble in deoxycholate and thence into the soluble fraction of the cell (Littlefield *et al.* 1955).

The specific activities of the proteins of the microsomal particles, of the microsomal material soluble in deoxycholate and of the soluble fraction of the cell from the livers of hypophysectomized rats are also shown in Fig. 1. It can be seen that the same relationship occurred between the specific activities of the proteins of the three subcellular fractions of the liver cell of hypophysectomized rats as between the same fractions from normal rat liver. It is clear, however, that the specific activity of the protein of the microsomal particles from livers of hypophysectomized rats is much less than that of the protein of these particles prepared from livers of normal rats for at least 10 min. after the injection of the radioactive amino acids. The specific activity of the protein of microsomal particles from normal rat liver falls more rapidly with time than does the specific activity of the proteins of the microsomal particles of livers of hypophysectomized rats, so that 20 min. after the injection of radioactive amino acids there is little difference between the specific activities of the proteins of the microsomal particles from the livers of differently treated rats.

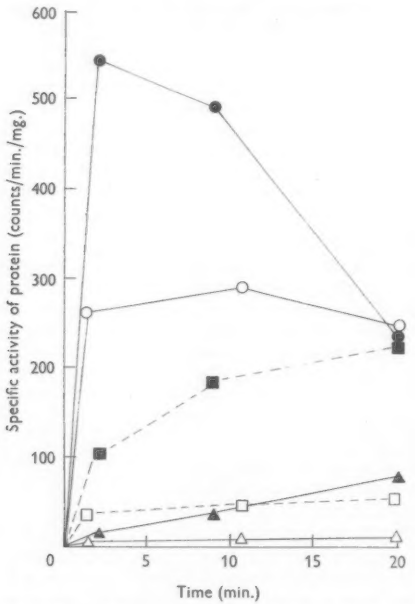

Fig. 1. Effect of hypophysectomy of the rat on the incorporation of intravenously injected L-[G-^{14}C]leucine into the proteins of the microsomal particles (●, ○), the proteins of that portion of the microsomes which is soluble in sodium deoxycholate (■, □) and the proteins of the soluble fraction of the liver cells (▲, △). Each point represents the mean of four experiments. Results from normal rats are indicated by filled-in points and those from hypophysectomized rats by open points.

Another difference between the normal and hypophysectomized rats, which is apparent from Fig. 1, is that the specific activity of the proteins of that portion of the microsomes which is soluble in deoxycholate increases at a much more rapid rate in normal rats than in rats which have been hypophysectomized. The specific activity of the proteins of the soluble fraction of the cell is also less in the hypophysectomized rats than in the normal ones (cf. Table 3).

Treatment of hypophysectomized rats with growth hormone tends to restore the extent of amino acid incorporation into protein towards that shown by normal rats and the distribution of radioactive protein between the microsomal particles, the deoxycholate-soluble material and the soluble fraction of the cell approaches that found with normal rats. Fig. 2 shows that the proteins of the microsomal particles have a greater specific activity in those hypophysectomized rats which were treated with growth hormone and that there is a more rapid fall in the specific activity of the proteins of these particles than in those of untreated hypophysectomized rats. At the same time the specific activity of the proteins of the deoxycholate-soluble portion of the microsomal particles rises more rapidly in the rats treated with growth hormone and so does the specific activity of the soluble-fraction protein.

It might appear from Fig. 1 that the specific activity of the proteins of the microsomal particles of livers from hypophysectomized rats does not reach its maximum value until about 10 min. after the injection of radioactive amino acids. An alternative explanation is that the maximum specific activity of microsomal-particle protein is reached only a little later in the hypophysectomized rats than in the normal or growth hormone-treated

hypophysectomized rats, but that it occurs after the first lobe of liver is removed from the hypophysectomized rats whereas the maximum specific activity is reached before the first lobe is removed from normal or growth hormone-treated hypophysectomized rats. Fig. 3 illustrates the results of an experiment which support this suggestion. In this experiment the first lobe of the liver was not removed from the hypophysectomized rat until 5 min. after the injection of radioactive amino acids, whereas, in contrast, the first lobe of the liver of the normal rat and of the hypophysectomized rat treated with growth hormone were removed as early as possible after the radioactive amino acid has been injected (0·7–1·2 min.). Here the maximum specific activity of the microsomal particle proteins has been reached by the time the first liver lobe was removed in the hypophysectomized rat but not in the normal rat nor the hypophysectomized rat which had been treated with growth hormone. In these experiments, the slow decline of the specific activity of the microsomal-particle protein from hypophysectomized rat liver was still apparent, as was the slow rise in the specific activity of the protein of the material soluble in deoxycholate.

It appears from Figs. 1 and 2 that hypophysectomy has caused a greater diminution of the specific activity of the protein of the deoxycholate-soluble fraction of the microsomes than can be accounted for solely on the grounds of the smaller specific activity of the proteins of the microsomal particles in these animals. In order to see if hypophysectomy and growth hormone treatment had, indeed, altered the ability of the synthesized polypeptides to peel off the microsomal template and be transferred to the lipoprotein part of the microsomes, the data of the experiments described in Figs. 1 and 2 were used to calculate the percentage of the total counts in the undivided microsomal protein which occurs in the proteins of the deoxycholate-soluble portion of the microsome (Table 4). The only differences which emerged occurred 20 min. after the injection of radioactive amino acids, when the percentage of the counts which appeared in the deoxycholate-soluble fraction from hypophysectomized rats was less than that in the same fraction from normal rats, or from hypophysectomized rats which had received growth hormone.

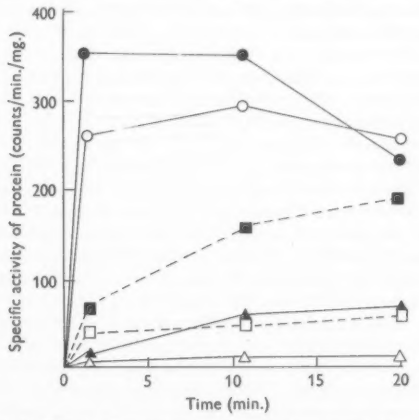

Fig. 2. Effect of treatment of hypophysectomized rats with 0·2 mg. of growth hormone/day for 7 days on the incorporation of intravenously injected L-[G-^{14}C]leucine into the protein of the microsomal particles (●, ○), the protein of that portion of the microsomes which is soluble in sodium deoxycholate (■, □) and the proteins of the soluble fraction of the liver cells (▲, △). Each point represents the mean of four experiments. Open points are used for results from hypophysectomized rats and filled-in points for hypophysectomized rats treated with growth hormone.

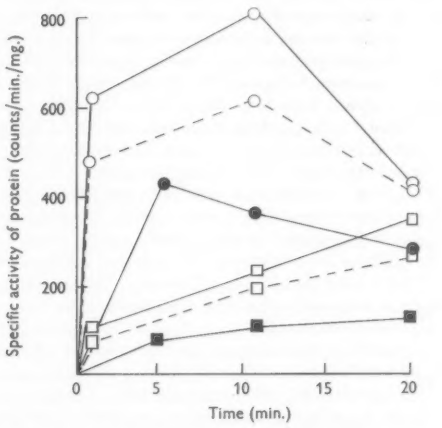

Fig. 3. Effect of hypophysectomy of the rat, and of treatment of hypophysectomized rats with 0·3 mg. of growth hormone/day for 5 days on the incorporation of intravenously injected DL-[1-^{14}C]leucine into the proteins of the microsomal particles of rat liver (●, ○) and into the deoxycholate-soluble fraction of the microsomes (■, □). In these experiments the first liver lobe of the normal rat and of the hypophysectomized rat given growth hormone were removed as soon as possible after injection of the amino acid, whereas the first lobe of the liver of the hypophysectomized rat was removed 5 min. after the injection. Results from normal rats are indicated by open points, and those from hypophysectomized rats by filled-in points. Results from hypophysectomized rats treated with growth hormone are indicated by open points and broken lines.

Table 4. *Effect of hypophysectomy of the rat, and of treatment of hypophysectomized rats with 0·2 mg. of growth hormone/day for 7 days, on the percentage of the total counts in the protein of the microsomes which occurs in the deoxycholate-soluble fraction of the microsomes, after intravenous injection of* L-[G-^{14}C]*leucine* (15 μc)

Data from the experiments described in Figs. 1 and 2.

Liver lobe	Time removed after injection of amino acid (min.)	Percentage of total counts/min. in microsome protein which occurs in the protein of the material soluble in deoxycholate (mean values; four rats in each group)		
		Normal rats	Hypophysectomized rats	Growth hormone-treated hypophysectomized rats
1	2	44	46	49
2	9–11	57	58	63
3	20	79	63	79

DISCUSSION

Growth hormone and protein synthesis. Experiments with cell-free preparations of rat liver have shown that amino acid incorporation into protein occurs in microsomes (Littlefield *et al.* 1955) and mitochondria (McLean, Cohn, Brandt & Simpson, 1958), and experiments with other tissues have shown that the nuclei are also capable of some protein biosynthesis (Allfrey, Mirsky & Osawa, 1957). The experiments reported in Tables 1 and 3 of this paper show that hypophysectomy inhibits, and treatment of hypophysectomized rats with various doses of growth hormone stimulates, protein synthesis in the liver *in vivo* overall, and in each of the particulate fractions of the liver cell. The experiments were short-term ones and it is highly unlikely that growth hormone can be acting solely by diminishing the amount of protein catabolism. These results lend support to the idea that the ability of growth hormone to enhance the rate of protein synthesis (see Russell, 1955) is responsible for much of the decrease in weight and protein content of rat liver which follows hypophysectomy, and the reversal of this trend in hypophysectomized animals treated with growth hormone (Simpson *et al.* 1949).

Few experiments have been reported on the influence of hypophysectomy, or of treatment with growth hormone, on the incorporation of radioactive amino acids into proteins, in the rat. Friedberg & Greenberg (1947) found that growth hormone increased the incorporation of [^{35}S]-methionine into muscle protein of hypophysectomized rats but that liver showed a reduction in amino acid uptake under the influence of growth hormone. They give no experimental evidence for this statement in the communication but cite as reference a thesis of the University of California which is not available to the author. Their experiments are open to the technical objection that methionine can be bound to protein by non-peptide linkages unless special precautions are taken in washing the protein while preparing it for radioactive counting (Tarver, 1954).

Lee & Williams (1952) studied the effect of growth hormone, administered to normal rats, on the incorporation of [^{35}S]cystine into nuclear, mitochondrial, microsomal and soluble fractions of rat liver. They reported that growth hormone did not alter the capacity of the liver of intact rats to incorporate cystine into protein, but that the incorporation into nuclear and mitochondrial fractions had increased as a result of the treatment with growth hormone.

The distribution of radioactive protein in these experiments (Friedberg & Greenberg, 1947; Lee & Williams, 1952) was examined several hours after administration of the labelled amino acid. Incorporation of amino acids into protein occurs very rapidly (Littlefield *et al.* 1955; Loftfield & Eigner, 1958) and the specific activity of the protein of those structures in the cell which are responsible for the assembly of amino acids into proteins will rise rapidly and fall again (if the intracellular amino acid specific activity drops) as the finished polypeptides are transferred from the template to other parts of the cell, or are secreted from the cell. The specific activities of proteins in other parts of the cell will also vary rapidly as a consequence of this shift of labelled material. The experiments reported in this paper were carried out with short time intervals between the administration of the radioactivity and the examination of the distribution of labelled protein in the cell. This difference in experimental design may help to explain the differences in the findings of Friedberg & Greenberg (1947) and Lee & Williams (1952), and those reported in this paper.

Balis, Samarth, Hamilton & Petermann (1958) found that treatment of intact male rats with growth hormone resulted in a more rapid incorporation of injected radioactive glycine into the pro-

teins of the microsomal particles of the liver. No rise in the extent of incorporation into these particles was noted after growth hormone treatment but this is not unexpected since the first time interval used was 15 min. after the injection of the radioactive amino acid, by which time much of any difference in specific activity between normal and treated rats would be expected to have disappeared (see Fig. 1). Balis *et al.* (1958) also reported that the specific activity of the proteins of the microsomal lipoprotein, combined with the protein of the soluble fraction of the cell, reached a maximum earlier than in untreated rats.

Ulrich, Tarver & Li (1954) found that hypophysectomy depressed the rate of turnover of serum albumin and that treatment of hypophysectomized rats with growth hormone restored the turnover rate almost to the normal level.

According to a recent personal communication, J. L. Kostyo and E. Knobil have observed diminished incorporation of injected radioactive leucine into tissue proteins after hypophysectomy. They found that administration of growth hormone to hypophysectomized rats increased amino acid incorporation only if the animals were fasted before injection of the radioactive amino acids. The rats used in most of the experiments described in this paper were effectively fasting for some hours before death (see Methods section), but a stimulating effect of growth hormone on amino acid uptake by tissues has also been noted in similar experiments carried out with fed rats. Fasting for 18 hr. did not alter the extent of incorporation of amino acids into protein in a system *in vitro* prepared from rat livers of normal or of hypophysectomized rats (Korner, 1959).

Location of the point of action of growth hormone. Some of the steps in the sequence of reactions which result in protein biosynthesis in the microsomal particles are known (see Hoagland *et al.* 1956; Hoagland *et al.* 1957; Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958; Littlefield *et al.* 1955). The control which is exerted by growth hormone, and by the other pituitary hormones, over the rate of biosynthesis of proteins could be exercised at one or more of these steps.

Size and composition of the free intracellular amino acid pool. The size of the amino acid pool inside the liver cell and the proportion of the amino acids of the pool which have arisen by entry into the cell, by controlled breakdown of tissue protein (Simpson, 1953; Korner & Tarver, 1957) or by synthesis of amino acids from carbohydrates, may well be under hormonal control. There is good evidence that growth hormone administered to the rat causes a fall in blood amino acid content (Griffith, Luck, Kulakoff & Mills, 1954; Li, Geschwind & Evans, 1949), and Russell (1955) has given some evidence that the size of the amino acid pool in rat liver is decreased by treatment with growth hormone. Schaffer & Lee (1935) found that anterior pituitary-gland extracts decreased the free amino acid content of many tissues of the rat, including the liver. Li *et al.* (1949) found that hypophysectomy resulted in a fall in blood amino acid level but that this low level was not depressed much further by treatment of hypophysectomized rats with growth hormone. Hypophysectomy also resulted in a decrease in the size of the free amino acid pool of rat liver (Schaffer & Lee, 1935).

The manner in which growth hormone causes alterations in the size of the amino acid pool in rat liver is not clear. No evidence is available on the effect of growth hormone on the rate of entry of amino acids into liver cells from the blood. Nor, indeed, is there any evidence that a permeability barrier to amino acids exists in liver cells. The rate of gluconeogenesis is controlled by adrenal steroids, but not by growth hormone, and the evidence that growth hormone controls protein catabolism is not very strong (see Ketterer, Randle & Young, 1957). It is probable that the observed fall in pool size and in blood amino acid content of rats treated with growth hormone is a secondary phenomenon which occurs as a result of the increased rate of protein biosynthesis caused by the growth hormone.

The methods used in this paper to measure the intracellular specific activity of the free amino acids revealed no significant differences between normal, hypophysectomized and growth hormone-treated hypophysectomized rats. The variation between the results of individual rats in each group was large and differences in specific activity between the groups might be revealed by experiments carried out on a larger scale. The tendency for the specific activity of the free amino acids of the liver of hypophysectomized rats to remain at or near the initial value whereas the values for normal and growth hormone-treated hypophysectomized rats fell from the initial high level might be a result of the more rapid incorporation of amino acids into protein both in the liver and in peripheral tissues of the rats which have growth hormone available.

It appears that the differences in amino acid incorporation into protein found to exist between normal rats, hypophysectomized rats and hypophysectomized rats given growth hormone cannot be ascribed entirely, or even in large measure, to hormonally controlled changes in the intracellular amino acid concentration, and therefore in the specific activity of the amino acids available at the site of protein synthesis.

It is, however, possible that much of the amino acid available in the cell for protein biosynthesis is not free but is bound to activating enzymes,

soluble RNA or other cell components. If this is so, effects of hormones on the size of the pool of amino acids effectively available for protein synthesis would not be revealed by measurements of the free amino acid content of the cell.

Ability of the soluble fraction to prepare amino acids for incorporation into protein. The diminished incorporation of amino acids into microsomal protein in a liver cell-free system which follows hypophysectomy was shown to be brought about by a change in the liver microsomes resulting from hypophysectomy (Korner, 1958, 1959). It was pointed out that hypophysectomy may also have decreased the ability of the soluble fraction of liver to prepare amino acids for incorporation into protein, either by a fall in the activity of the amino acid-activating enzymes, or by a change in the rate of formation of the amino acid–soluble RNA intermediate, but that such changes may not be limiting and so would not be detected in the system used *in vitro*. In the experiments described in this paper, the lower maximum specific activity of the liver microsomal-particle protein of hypophysectomized rats can be ascribed to some change in the particles, resulting from hypophysectomy, which diminishes their ability to incorporate amino acids into proteins. The slower rise to the maximum specific activity shown by these particles from hypophysectomized rats may also be caused by some change in the particle which makes it more difficult for the activated amino acids to enter the particle or to find their correct locus on the RNA template. On the other hand, it is possible to argue that the cause is a slower preparation of the amino acids for incorporation into protein. Hypophysectomy may have caused a fall in the activity of the amino acid-activating enzymes, in the ability to form an amino acid–soluble RNA complex, in the ability to transfer the amino acid from the complex to the microsomal particle or in the supply of adenosine triphosphate or guanosine triphosphate needed for these processes.

Formation of polypeptide chains in the microsomal particle. The experiments described in Figs. 1–3 of this paper show that hypophysectomy has resulted in a reduced ability of the microsomal particles of rat liver to assemble amino acids into polypeptide chains. Treatment of the hypophysectomized rat with growth hormone can, to some extent, restore towards normal this reduced ability to synthesize protein. The other pituitary-gland hormones are probably also involved in the control of protein biosynthesis, for treatment of hypophysectomized rats with growth hormone alone has not succeeded in completely reversing the effect of hypophysectomy. This finding confirms the conclusions drawn from experiments *in vitro* (Korner, 1958, 1959).

Transfer of the synthesized peptide from the template to the lipoprotein of the endoplasmic reticulum. Twenty minutes after an injection of radioactive amino acids, a lower proportion of the total counts of the microsomal protein occurs in the lipoprotein portion of the endoplasmic reticulum in rats which have been hypophysectomized than is the case in normal rats, or in hypophysectomized rats treated with growth hormone (Table 4). This difference may be caused by a diminished ability of the synthesized polypeptides to peel off the RNA template of microsomal particles of livers of hypophysectomized rats. However, the effect is not manifest at times earlier than 20 min. after the injection of the amino acids and it is possible that the difference is not caused by any difference in the ability of the peptides to come off the template but is merely an effect of the maintenance of the specific activity of the intracellular amino acids at a high level for a longer time in the hypophysectomized rats than in the normal rat, which leads to the slower decline in the specific activity of the microsomal-particle protein and thus to a lower percentage of the counts in the deoxycholate-soluble protein of the livers of hypophysectomized rats.

Littlefield *et al.* (1955) showed that when high doses of radioactive amino acids were injected into rats, the specific activity of the microsomal-particle protein remained at a high level and did not fall off as was the case in experiments in which small 'pulse' doses of radioactivity were administered to rats. The slight tendency for the microsomal-particle protein from hypophysectomized rat liver to show this levelling off (Figs. 1 and 2) may be partly a result of the slower fall in the specific activity of intracellular free amino acids (Table 2), thus giving an apparently 'high dose' type of experiment.

SUMMARY

1. The incorporation *in vivo* of injected radioactive amino acids into the total protein of rat liver and into the proteins of the nuclei, mitochondria, microsomes and soluble fraction is diminished by hypophysectomy. Treatment of hypophysectomized rats with ox growth hormone restored, in part, the diminished incorporation of amino acids into the total proteins and into the proteins of the subcellular fractions of rat liver, towards the normal values. These changes in the amount of amino acid incorporation into proteins could not be ascribed to hormonally controlled changes in the specific activity of the intracellular amino acid pool.

2. Separation of the microsomes into microsomal particles and into material soluble in deoxycholate showed that there was a diminished

ability of the microsomal particles of liver from hypophysectomized rats to incorporate amino acids into protein. The maximum specific activity of the protein of these particles was depressed as a result of hypophysectomy and, in addition, this maximum value was attained at a longer time interval after injection of the radioactive amino acids than was the case with normal rats. Treatment of the hypophysectomized rats with growth hormone reversed both these trends to some extent.

3. The possible effects of hypophysectomy of the rat and of treatment of the hypophysectomized rat with growth hormone on different stages of protein synthesis are discussed.

It is a pleasure to thank Professor F. G. Young, F.R.S., for his interest in this work and for his encouragement. I wish to thank the Council of the Royal Society for a Grant-in-aid, Miss M. B. Thomas who performed the hypophysectomies and Mr D. C. Gardiner for highly skilled assistance.

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The Effect of the Administration of Insulin to the Hypophysectomized Rat on the Incorporation of Amino Acids into Liver Proteins *in vivo* and in a Cell-Free System

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It is generally accepted that insulin is a protein anabolic hormone. The rate of protein biosynthesis, measured in several ways, is subnormal in tissues of diabetic animals and can be restored towards the normal by treatment of the animals with exogenous insulin (see reviews by Lukens, 1954; Lukens & McCann, 1955 and Krahl, 1956). Some workers consider that insulin can exert its anabolic action in the rat in the absence of growth hormone (Salter & Best, 1953; Lawrence, Salter & Best, 1954; Best, 1955; Salter, Davidson & Best, 1957; Griffiths, 1956; Lukens, 1958), but Wagner & Scow (1957) believe that exogenous insulin acts as a growth stimulant in hypophysectomized rats only by causing an increase in their food intake.

It has been shown that hypophysectomy of the rat reduces the amount of injected radioactive amino acid which is incorporated *in vivo* into the total liver proteins, and into the proteins of the subcellular fractions of the liver (Korner, 1960). It has also been shown (Korner, 1959) that hypophysectomy of the rat results in a diminished incorporation of radioactive amino acids into microsomal protein *in vitro* in a liver cell-free system of the type described by Zamecnik & Keller (1954). Treatment of the hypophysectomized rat with growth hormone could, to a large extent, reverse these effects of hypophysectomy.

Experiments are described in this paper in which the effects of insulin treatment of hypophysectomized rats on the subsequent incorporation of amino acids into liver proteins *in vivo*, and in a cell-free system *in vitro*, have been studied. A preliminary report of some of this work has been published (Korner, 1958).

METHODS

Animals. Young, adult, male and female albino rats, of the same age and weight, and of an inbred strain, were used. They were hypophysectomized under ether anaesthesia by the parapharyngeal approach (Ingle & Griffith, 1942). After recovery from the operation each rat was housed in an individual cage and all the rats used in an experiment were given the same ration of a powdered diet (diet 41, Bruce & Parkes, 1949) made into a thick paste with water. The daily ration, established by trial and error, was the largest quantity of food which was entirely consumed by every rat (approx. 10 g./100 g. of rat/day). Water was freely available. The animals were kept on this ration for 3 weeks before being used in an experiment. The rats were not starved on the night before they were killed but each rat had eaten its last ration several hours before it was killed. From the time injections with insulin were begun, 5% glucose soln. was made available to all the rats in an experimental group.

Insulin. Protamine zinc insulin B.P. (Burroughs Wellcome and Co.), 40 i.u./ml., was used in most experiments. It was diluted with 0·9% NaCl so that the appropriate dose could be given by subcutaneous injection in a volume of 0·5 ml. Control rats were given 0·5 ml. of 0·9% NaCl by subcutaneous injection.

Samples of six-times recrystallized insulin, obtained from Boots Pure Drug Co. Ltd., or samples of glucagon-free insulin, obtained from Novo Terapeutisck Laboratorium, were used in some experiments. The samples of crystalline insulin were dissolved in 3 mN-HCl and the solution was diluted with 0·9% NaCl to the required concentration.

Radioactive compounds. Generally (G) labelled L-[G-^{14}C]-leucine, DL-[1-^{14}C]leucine, DL-[1-^{14}C]valine and hydrolysed algal protein labelled with ^{14}C were used and were obtained from The Radiochemical Centre, Amersham, Bucks. For administration *in vivo* they were dissolved in 0·9% NaCl so that the appropriate dose could be administered in a volume of 0·5 ml.; for experiments *in vitro* the radioactive amino acids were dissolved in the medium used for incubation (see below).

Materials. The sodium salts of adenosine triphosphoric acid (ATP) and of guanosine triphosphoric acid were obtained from the Sigma Chemical Co. The sodium salt of creatine phosphate was prepared by the method of Ennor & Stocken (1948) and creatine phosphokinase was prepared by the method of Kuby, Noda & Lardy (1954).

Experimental procedure for experiments in vivo. The radioactive amino acids were injected into the femoral vein of the rat, which had been anaesthetized with Nembutal, and three lobes of the liver were removed at successive intervals (about 2, 8 and 20 min.) after the injection of the amino acids, in the way previously described (Korner, 1960). The lobes of liver were placed in ice-cold 0·35 M-sucrose containing an excess of unlabelled amino acid, corresponding to the radioactive amino acid injected, and the liver was minced with scissors. The tissue was homogenized and separated into nuclei, mitochondria, microsomes and soluble fraction of the cell by differential centrifuging in the way previously described (Korner, 1960). These cell fractions were washed with sucrose and their proteins were precipitated with 0·5 N-perchloric acid. In some experiments the microsomal pellet was treated with sodium deoxycholate (Littlefield, Keller, Gross & Zamecnik, 1955; Palade & Siekevitz, 1956) in order to separate the ribonucleoprotein microsomal particles from the lipoprotein, deoxycholate-soluble fraction of the microsomes, in the manner described previously (Korner, 1960). The extent to which this separation had been achieved was determined by estimation of the ribonucleic acid (RNA)/protein ratio of the microsomal particles. This ratio was between 0·6 and 0·85 (cf. Littlefield *et al.* 1955; Palade & Siekevitz, 1956).

Experimental procedure for the experiments in vitro. The rats were killed by decapitation and were bled. The liver was rapidly removed and cut up with scissors into an ice-cold homogenizing medium consisting of 0·03 M-2-amino-2-hydroxymethylpropane-1:3-diol hydrochloride (tris) buffer, pH 7·5, containing 0·08 M-KCl, 0·05 M-NaCl, 5 mM-$MgCl_2$ and 25 mM-glutathione. The pH was measured with a glass electrode which had been standardized with a standard buffer soln. made in accordance with the manufacturers' instructions from Burroughs Wellcome and Co. N.B.S. buffer solution tablets. The standard solution was pH 6·99 at 25°. A portion of the liver was blotted, weighed by displacement in ice-cold medium and homogenized in 5 vol. of the medium in a hand-operated homogenizer of the type described by Dounce, Witter, Monty, Pate & Cottone (1955), which was kept cold in an ice bath. Only three strokes (up and down) were used since severe damage to the tissues diminishes its capacity to incorporate amino acids *in vitro* (Zamecnik & Keller, 1954).

The resulting brei was spun at 15 000 *g* for 10 min. at 0° in a refrigerated centrifuge to remove unbroken cells, cell debris, nuclei and mitochondria. The supernatant fluid was carefully removed from the pellet and was spun at 105 000 *g* for 1 hr. at 0° in a Spinco model L preparative ultracentrifuge. The soluble fraction of the cell was carefully removed from the microsome pellet and the pellet and tube were rinsed in the tris medium and the tube was blotted with tissue paper. The microsome pellet was suspended by gentle homogenization in fresh tris homogenizing medium and made up to a known volume. Great care was taken to standardize rigidly the method of preparation of the microsomes and the soluble fraction of the cell, so that they

would be prepared in exactly the same way from differently treated rats.

Sachs (1957) has shown that the cell-free system prepared from rat liver can be freeze-dried and stored at −20° without loss of the ability to incorporate amino acids into protein *in vitro*. In some experiments liver material which had been stored in the freeze-dried state was used. It was prepared in the manner described previously (Korner, 1959).

Conditions of incubation. Samples of the microsomal and soluble fractions of the cell, containing the required amounts of protein, were shaken at 37° for various times in the presence of 1 mM-ATP, 0·02 M-creatine phosphate, 0·6 mM-guanosine triphosphate, 0·03 mg. of creatine phosphokinase and 0·4 μC of radioactive amino acid/ml. of final volume in a total volume of 1 ml. Each solution was adjusted to pH 7·5 with 0·1 N-KOH before it was added to the mixture. The reaction was stopped by addition of cold 0·5 N-perchloric acid, or, if the microsomes were to be removed from the soluble fraction, by pouring the contents of each tube into a cooled Spinco centrifuge tube containing ice-cold 0·25 M-sucrose, together with an excess of unlabelled amino acid. The tubes were then centrifuged at 105 000 *g* for 1 hr. The proteins of the soluble fraction and of the resuspended microsomes were precipitated with cold 0·5 N-perchloric acid.

Preparation of radioactive proteins. The precipitated protein was washed, RNA and lipid were extracted, the residual protein was dried and plated on to weighed planchets and the radioactivity assessed by counting in a Geiger–Müller end-window (1·5–2·5 mg./ml.) counter in the manner previously described (Korner, 1959). Sufficient counts were recorded to reduce the standard error to 3% or less and corrections were made for the background counts and for self-absorption. The results are given as the specific activity of the protein (counts/min./mg. of protein).

Determination of ribonucleic acid. The RNA content of samples was determined by the slight modification of the method of Scott, Fraccastoro & Taft (1956), which has been previously described (Korner, 1959).

Determination of protein content. The N content of replicate samples of the microsomes and of the soluble fraction which were incubated in the experiments *in vitro*, or of the fractions of liver separated after the experiments *in vivo*, were determined by the micro-Kjeldahl technique. These values of N were corrected for the N content of any tris buffer present in the sample, and for the N content of the RNA of the sample (14% being used as the N content of RNA). The protein content of the samples was calculated from the corrected N figure by use of the factor 6·25. In some of the experiments *in vitro* the specific activity of the microsomal protein was calculated from the expression $(SP/M).(X/100)$, where S is the specific activity of the total protein isolated, P is the wt. (mg.) of protein incubated, M is the wt. (mg.) of microsomal protein incubated and X is the percentage of the total radioactivity in the protein of the sample which is found to be present in the microsomal protein; X was determined in the manner previously described (Korner, 1959). When results are presented which have been calculated by use of this expression a note to this effect appears in the legend to the table or figure.

Statistical treatment of results. Each experiment was repeated several times with rats kept under identical conditions and the mean of the experimental observations, together with the standard error of their means (S.E.M.), was calculated. Student's t test was applied to ascertain if the difference between mean values was statistically significant, and the values of Fisher's P are given when such a difference is considered to be statistically significant.

RESULTS

Experiments in vivo. Table 1 records the specific activities, after an intravenous injection of 15 μC of DL-[1-^{14}C]leucine, of the proteins of the nuclei, mitochondria, microsomes and soluble fraction of the three lobes of liver of hypophysectomized rats and of insulin-treated hypophysectomized rats. It will be seen that more radioactive amino acid has been incorporated into the proteins of the mitochondria, microsomes and soluble fraction in the livers of the insulin-treated rats than into the proteins of these subcellular liver fractions of untreated rats. The individual data for nuclei are too scattered for any conclusion to be drawn about the effect of insulin treatment of hypophysectomized rats on amino acid incorporation into the proteins of this subcellular fraction.

The distribution of radioactive protein in the ribonucleoprotein microsomal particles and in the lipoprotein, deoxycholate-soluble fraction of the

Table 1. *Effect of exogenous insulin administered to female hypophysectomized rats on the incorporation of injected* DL-[1-^{14}C]*leucine into the proteins of nuclei, mitochondria, microsomes and soluble fraction of the three lobes of liver*

Lobes 1–3 were removed 2·5, 8 and 20 min. respectively after the injection of radioactive amino acids. Protamine zinc insulin (2 i.u./day) was given for 5 days.

		Specific activity of protein (mean ± S.E.M.; four rats in each group)			
	Liver lobe	Nuclei	Mitochondria	Microsomal	Soluble fraction
Hypophysectomized rats	1	30 ± 3·9	25 ± 2·6	172 ± 16·5	16 ± 3·6
	2	41 ± 5·1	49 ± 4·3	251 ± 24·2	36 ± 2·9
	3	58 ± 5·2	78 ± 5·1	340 ± 33·9	41 ± 4·5
Insulin-treated hypophysectomized rats	1	27 ± 3·1	37 ± 3·9	244 ± 21·5	24 ± 2·1
	2	55 ± 4·9	69 ± 5·1	425 ± 39·6	47 ± 3·1
	3	64 ± 4·8	97 ± 6·4	410 ± 39·1	69 ± 4·9

microsomes, after injection of L-[G-^{14}C]leucine, are shown in Fig. 1. In the normal rats the specific activity of the protein of the microsomal particles rises rapidly to a maximum value and then decreases (Littlefield *et al.* 1955; Korner, 1960). Meanwhile there occurs a slower but maintained rise in the specific activity of the protein of the deoxycholate-soluble portion of the microsome together with a slow rise in the specific activity of the soluble fraction of the cell. This distribution of radioactive protein in these subcellular fractions is in keeping with the idea that the amino acids are assembled into polypeptide chains on a RNA template in the microsomal particles and that the newly synthesized polypeptide passes to that part of the microsome which is soluble in deoxycholate, and thence to the soluble fraction of the cell (Littlefield *et al.* 1955). The ribonucleoprotein particles from livers of hypophysectomized rats show a slower rise to the maximum value of specific activity as well as a lower maximum value. The specific activity of the microsomal particles decreases more slowly than in normal rats and there is a slower rise in the specific activity of the proteins of the deoxycholate-soluble fraction of the microsome and of the soluble fraction of the cell (Korner, 1960). It can be seen from Fig. 1 that treatment of hypophysectomized rats with insulin has restored the distribution of radioactivity towards that found in normal rats. Insulin treatment of hypophysectomized rats has increased the ability of the microsomal particles to incorporate amino acids into proteins and this is reflected in the higher specific activity of the proteins of the particles prepared from the livers of insulin-treated hypophysectomized rats. In addition, the maximum specific activity of the proteins of the microsomal particles is reached more quickly in the insulin-treated rats than in the untreated hypophysectomized rats, for the treated rats showed maximum specific activity of the proteins of the particles when the first liver lobe was removed, whereas the particle protein of untreated rats had not yet reached its maximum value at this time. The specific activity of the proteins of the microsomal particles from insulin-treated hypophysectomized rats falls more rapidly from the maximum value than is the case with particles from the untreated animals. It can also be seen from the graph that insulin treatment has resulted in a more rapid rise in specific activity of the proteins of the deoxycholate-soluble material and of the soluble proteins of the cell. Similar results to the ones described here have been obtained in experiments where hydrolysed algal [^{14}C]protein was injected into the rats.

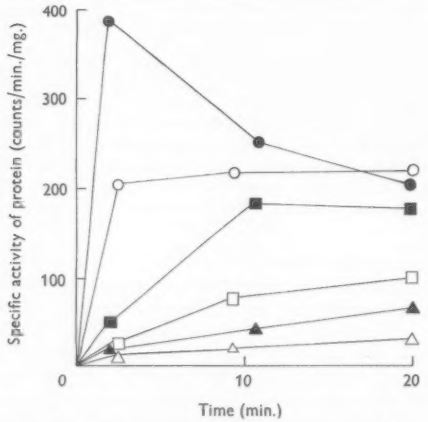

Fig. 1. Effect of treatment of hypophysectomized female rats with protamine zinc insulin (3 i.u./day for 5 days) on the incorporation of intravenously injected L-[G-^{14}C]-leucine (12 μc) into the proteins of the microsomal particles (●, ○), the protein of the deoxycholate-soluble fraction of the microsomes (■, □) and the proteins of the soluble fraction of the cell (▲, △). Each point represents the mean of four experiments. Results from hypophysectomized rats are denoted by open points, those from insulin-treated hypophysectomized rats by filled-in points.

Table 2. *Effect of insulin treatment of hypophysectomized rats on the percentage of the total counts in microsomal protein, which are present in the deoxycholate-soluble fraction of the microsome at various times after an injection of* L-[G-^{14}C]*leucine*

Protamine zinc insulin (3 i.u./day) was given for 5 days. Data are from the experiment described in Fig. 1.

Liver lobe	Percentage of total counts/min. in the microsomal protein which occurs in the deoxycholate-soluble fraction (mean of four experiments ± S.E.M.)	
	Hypophysectomized rats	Insulin-treated hypophysectomized rats
1	38·5 ± 1·4	39 ± 1·7
2	64 ± 3·1	78 ± 2·9
3	69 ± 3·4	82 ± 3·0

Table 2 shows the percentage of the total counts of the microsomal protein which occurs in the deoxycholate-soluble fraction of the microsomes after the injection of L-[G-^{14}C]leucine. (A higher proportion of the total radioactive protein is present in the lipoprotein fraction in the insulin-treated rats, except at the earliest time interval after the injection of the radioactive amino acids.)

Experiments in vitro. Table 3 gives the results of an experiment *in vitro* in which microsomes and soluble fraction of liver were incubated with DL-[1-^{14}C]leucine. It is clear that treatment of female

hypophysectomized rats with 2 i.u. of protamine zinc insulin/day for 4 days resulted in a 44% rise in the amount of amino acid incorporated into microsomal protein, compared with that incorporated into microsomal protein in the system prepared from livers of untreated hypophysectomized rats. The difference between the mean values of the specific activities of the microsomal protein from treated and untreated rats is significant statistically ($P < 0.01$). Most of the increased incorporation resulting from insulin treatment of the hypophysectomized rats results from an increased ability of the microsomes from insulin-treated rats to incorporate amino acids into protein, for when microsomes from insulin-treated rats were incubated with soluble fraction from liver of untreated rats, almost as much amino acid is incorporated into proteins as when both parts of the system were prepared from insulin-treated animals. Some increase in the ability of the soluble fraction to prepared amino acids for incorporation is, however, apparent as a result of insulin treatment, for more amino acid is incorporated into protein when microsomes from livers of untreated rats are incubated with soluble fraction from livers of insulin-treated rats, than when the microsomes from untreated rats are incubated with soluble fraction prepared from livers of untreated rats. Although these differences are small and are not statistically significant they have been observed consistently in several other experiments of this type.

The response to insulin treatment of hypophysectomized rats can be detected as early as 3 hr. after an injection of 2 i.u. of crystalline insulin (Table 4). One hour after insulin injection there appears to be a small decline in the ability of the cell-free system to incorporate amino acids. It was shown by experiments of the type illustrated in Table 3 that this depression in incorporating ability was caused by alterations in the soluble fraction of the cell which rendered it less active in preparing amino acids for incorporation into proteins. It will be seen from Table 4 that male hypophysectomized rats are more sluggish in their anabolic response to insulin than female rats, for the earliest rise detected in the incorporating ability of the cell-free system prepared from male rats was 6 hr. after the insulin injection, whereas a rise was noted 3 hr. after injection of insulin with the female rats. In addition, the percentage increase in amino

Table 3. *Incorporation of* DL-[1-^{14}C]*leucine into microsomal protein in a cell-free system*

Microsomes and soluble fraction were prepared from livers of female hypophysectomized rats, some of which had been treated with insulin, as shown. Microsomal protein (3·5 mg.) and 4 mg. of soluble-fraction protein were incubated for 30 min. at 37° in 0·03 M-tris buffer, pH 7·5, containing 0·08 M-KCl, 0·05 M-NaCl, 5 mM-$MgCl_2$, 2·5 mM-glutathione, 1 mM-ATP, 0·01 M-creatine phosphate, 0·6 mM-guanosine triphosphate, 0·03 mg. of creatine phosphokinase and 0·4 μC of DL-[1-^{14}C]leucine in a total volume of 1 ml.

Microsomes from hypophysectomized rats which had received	Soluble fraction from hypophysectomized rats which had received	Specific activity of microsomal protein (counts/min./mg.) (mean of eight experiments ± S.E.M.)
No insulin	No insulin	84 ± 8·6
Protamine zinc insulin (2 i.u./day for 4 days)	Protamine zinc insulin (2 i.u./day for 4 days)	121·5 ± 10·2
No insulin	Protamine zinc insulin (2 i.u./day for 4 days)	97 ± 7·3
Protamine zinc insulin (2 i.u./day for 4 days)	No insulin	112 ± 8·7

Table 4. *Incorporation of* DL-[1-^{14}C]*leucine into microsomal protein in a cell-free system prepared from livers of hypophysectomized rats at various periods after injection of 2 i.u. of insulin*

Conditions were as described in the legend to Table 3. The specific activities were calculated from the expression discussed in the Methods section.

Time (hr.) after administration of 2 i.u. of crystalline insulin	Female rats		Male rats	
	Specific activity of microsomal protein	Percentage gain over control	Specific activity of microsomal protein	Percentage gain over control
Nil	56·6	—	64·0	—
1	55·0	−2·8	56·2	−12·5
3	66·8	18·0	69·0	4·7
6	83·0	46·6	77·5	20·5
24	79·6	40·6	82·0	28·0

acid incorporation resulting from insulin treatment is lower in male rats than in female rats. A similar sex difference in response to insulin has been noted in intact rats (A. Korner, unpublished work).

DISCUSSION

Insulin and growth of hypophysectomized rats. Insulin, administered to intact rats, induces a rise in the ability of rat-liver microsomes to incorporate amino acid into protein in a cell-free system *in vitro* (A. Korner, unpublished work). The results of the experiments set forth in this paper indicate that insulin is able to do this in the absence of pituitary gland, and that stimulating effects of treatment of hypophysectomized rats with insulin on the amino acid incorporation into protein *in vivo* can be shown in whole animals.

These experiments support the view that insulin can stimulate protein synthesis in the rat in the absence of growth hormone. Salter & Best (1953) and Lawrence *et al.* (1954) found that insulin, administered to hypophysectomized rats, stimulated a gain in body weight, an increase in width of the epiphyseal cartilage and some accumulation of protein. These observations were confirmed by Griffiths (1956). A gain in weight did not occur in hypophysectomized, alloxan-diabetic rats treated with growth hormone, but a weight gain was noted when insulin was given to these animals (Best, 1955; Salter *et al.* 1957). Wagner & Scow (1957) found that insulin caused no increase in the gain in weight and in protein content shown by hypophysectomized rats when they were force-fed. They believe that the weight gain observed in insulin-treated hypophysectomized rats by Salter & Best (1953) and Lawrence *et al.* (1954) is caused only by the increased food intake of the treated animals, and is not a manifestation of a direct stimulating action of insulin on protein anabolism.

Manchester, Randle & Young (1959) have found that insulin, added *in vitro*, stimulates the incorporation of radioactive amino acids into the proteins of the isolated diaphragm of hypophysectomized rats. They have shown that added insulin can cause this increased incorporation of amino acids into diaphragm proteins even when no glucose is present in the incubation medium. These observations are support for the view that insulin is a hormone concerned with anabolism of protein which is capable of stimulating protein synthesis both in the absence of growth hormone and, also, in a manner which is not connected with its known effects on the uptake of glucose by muscle cells.

Salter *et al.* (1957) found that insulin did not stimulate growth in hypophysectomized rats unless they were allowed to eat more food than they were eating before insulin treatment. In the experiments described in this paper, glucose solution was made available to all rats used in an experiment as soon as insulin injections were begun in the experimental group. The glucose intake of the insulin-treated rats and of their control rats is, however, not known.

Comparison of the effect of insulin and of growth hormone on protein synthesis in hypophysectomized rats. Evidence has been produced that in the cat and dog growth hormone cannot exert its anabolic effects unless extra insulin is made available to the animal (Gaebler & Robinson, 1942; Milman, de Moore & Lukens, 1951; Gaebler, Liu & Zuchlewski, 1956; Lukens & McCann, 1955). In the hypophysectomized–pancreatectomized rat, on the other hand, growth hormone can exert its anabolic action without the administration of more insulin than is needed to control the diabetes (Scow, 1957; Scow, Wagner & Ronov, 1958). Young (1953) has suggested that growth hormone exerts some, at least, of its anabolic effects by its ability to stimulate the secretion of insulin (see Ketterer, Randle & Young, 1957, for review; Engel, Albertson, Fredericks & Lopez, 1958). It is, however, probable that growth hormone has some other anabolic action apart from its ability to stimulate the secretion of insulin, for the results of Salter *et al.* (1957) show that hypophysectomized rats respond in a different way, both qualitatively and quantitatively, to treatment with growth hormone than to treatment with insulin.

Hypophysectomy of the rat brings about a fall in the size and protein content of rat liver (Simpson, Evans & Li, 1949). These changes found after hypophysectomy are probably caused by a fall in the ability of rat liver to synthesize protein (Russell, 1955). It has been shown that hypophysectomy of the rat causes a fall in the ability of rat liver to incorporate amino acids into protein, both *in vivo* (Korner, 1960) and in a cell-free system (Korner, 1959). Evidence was produced in these papers that hypophysectomy causes an alteration in the microsomes of rat liver such that the rate of protein biosynthesis in them is depressed. It was shown that treatment of hypophysectomized rats with growth hormone restored the ability to synthesize protein towards the rate found in normal rat liver.

It is clear from the experiments described in this paper that insulin, like growth hormone, is also capable of stimulating the depressed activity of the protein-synthesizing system of liver of rats which have been hypophysectomized. Insulin, like growth hormone, brings about a rise in the ability of microsomes from livers of hypophysectomized rats to assemble amino acids into protein in the cell-free system and *in vivo*. Unlike growth hormone, however insulin also appears to increase the

ability of the soluble part of the cell to prepare amino acids for incorporation into proteins in the system *in vitro*.

This improved ability to prepare amino acids for incorporation in the system *in vitro* could not have been caused by any change which may have occurred in the energy-producing mechanism of the cell as the result of insulin treatment, since the conditions of incubation are such that excess of energy is available throughout the life of the system, but insulin may well have caused alterations in the energy-producing mechanisms in the living animal.

Further work must be carried out to find out whether the insulin stimulated improvement in the ability of the soluble fraction of liver to prepare amino acids for incorporation into protein is caused by an alteration in the size of the intracellular amino acid pool, in the activity of the amino acid-activating enzymes (Hoagland, Keller & Zamecnik, 1956), in the ability to form an amino acid-soluble RNA intermediate (Hoagland, Zamecnik & Stephenson, 1957) or in the ability to transfer amino acids from this intermediate to the microsomal particles (Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958).

Another difference between the ways in which treatments with insulin and growth hormone of hypophysectomized rats bring about the greater incorporation of amino acid into protein in the cell-free system is that the effect of insulin treatment can be detected earlier than that of growth hormone. Insulin causes positive effects which can be detected 3 hr. after injection but growth hormone needs about 20 hr. before causing positive effects measurable by this system (Korner, 1959). Neither treatment restores entirely the depressed rate of amino acid incorporation into proteins shown by the liver of hypophysectomized rats.

The differences in response of hypophysectomized rats to treatment with growth hormone or with insulin lend support to the idea that growth hormone exerts anabolic effects apart from its ability to stimulate insulin secretion.

As with treatment with growth hormone, insulin might appear to be facilitating the release of newly synthesized polypeptides to the lipoprotein deoxycholate-soluble fraction of the microsomes, from the ribonucleoprotein templates of the microsomal particles (Table 2), but it is possible to explain the findings in other ways (cf. Korner, 1960).

Sex difference in the response to insulin. The greater responsiveness of female rats, compared with male rats, to the stimulating effects of insulin on protein anabolism may perhaps be explained by the presence of the anabolic stimulus of the androgens in male rats. Some of the possible stimulation of protein synthesis may already have occurred as a result of the presence of androgens.

SUMMARY

1. Hypophysectomized rats treated with insulin incorporated more of an intravenously administered dose of radioactive amino acids into the proteins of liver mitochondria, microsomes and soluble fraction than did untreated hypophysectomized rats.

2. The distribution of radioactivity in the microsomal particles, and in the portion of the microsomes soluble in deoxycholate, indicates that treatment with insulin tends to restore the diminished ability of the microsomal particles of the liver of hypophysectomized rats to take up amino acids and assemble them into polypeptide chains. Insulin treatment of hypophysectomized rats diminishes the time elapsing before the maximum specific activity of the protein of the ribonucleoprotein microsomal particles is reached.

3. Experiments with a cell-free system *in vitro* confirms that insulin treatment of hypophysectomized rats enhances the diminished ability to incorporate amino acids into protein shown by the liver microsomes. The results also suggest a small rise in the ability of the soluble fraction of liver from hypophysectomized rats to prepare amino acids for incorporation into proteins.

4. Cell-free preparations from the livers of male and of female hypophysectomized rats showed that the female hypophysectomized rats had responded more quickly and to a greater extent to the stimulating effects of insulin treatment than male hypophysectomized rats.

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Aminoacetone Formation by *Staphylococcus aureus*

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It has been reported (Elliott, 1958) that washed cell suspensions of *Staphylococcus aureus* are capable of producing a previously unknown amino compound which, provisionally identified as aminoacetone, was found to arise by two apparently distinct metabolic pathways. The first of these required the presence of threonine and oxygen whereas the second required the presence of glycine, glucose and oxygen. The present paper describes in detail the formation of the new metabolite, its isolation as a crystalline derivative and identification as aminoacetone. The possible role of the compound in metabolism is also discussed.

A preliminary account of this work has already been published (Elliott, 1959).

MATERIALS

δ-Aminolaevulinic acid hydrochloride was a Parke, Davis and Co. Ltd. product, kindly given by Dr J. E. Falk of C.S.I.R.O., Canberra. Aminoacetone hydrochloride and aminoacetone toluene-*p*-sulphonic acid salt were synthesized as described by Elliott (1960). DL-β-Hydroxyglutamic acid, DL-homoserine and DL-*allo*threonine were L. Light and Co. Ltd. products.

Phosphate buffers were mixtures of Na_2HPO_4 and KH_2PO_4. Phosphotungstic acid was purified by the method of Van Slyke, Hiller & Dillon (1942).

METHODS

Estimation of aminoacetone

The picrate-reaction method of Shuster (1956) as modified by Elliott (1960) was used. In experiments in which aminoacetone formation from threonine was measured, determinations were carried out directly on the incubation media after removal of the cells by centrifuging. When aminoacetone formation from glycine was measured it was necessary to concentrate the metabolite by treatment on Dowex 50 columns as described previously (Elliott, 1960).

Paper chromatography

Whatman no. 3 papers were used throughout and washed chromatographically by allowing water to drip off the bottom of the sheets for 3 days.

Solvent systems. (I) 2-Methylpropan-2-ol–methyl ethyl ketone–formic acid–water (120:50:40:40); (II) ethanol–

M-acetic acid–pyridine–water (95:10:3:3); (III) redistilled mesityl oxide–85% formic acid–water (1:1:2) (Lugg & Overell, 1948); (IV) butan-1-ol–acetic acid–water (4:1:5). The upper layers of mixtures (III) and (IV) were used.

Colour reactions on paper

The following colour reactions were used for the detection of aminoacetone.

Ninhydrin. Papers were dipped in 0·2% ninhydrin in acetone solution and then heated in an oven at 80°.

Copper–ninhydrin. Papers were dipped in a solution containing 0·4 ml. of aqueous saturated solution of $Cu(NO_3)_2$ in 100 ml. of acetone. After drying, ninhydrin was applied as described above. α-Amino acids do not give ninhydrin colours after copper treatment whereas other amino compounds still react.

Picrate spray. Chromatograms were sprayed on both sides of the paper with a mixture containing picric acid (saturated solution), water and NaOH (10N), in the proportions of 10:50:20. After 1 min. the papers were sprayed lightly with 5N-HCl. Aminoketones give orange–brown spots.

Modified Elson–Morgan reaction. Papers were sprayed with a mixture containing acetate buffer (M, pH 4·6) and acetylacetone (10:1, v/v), heated in an autoclave for 3 min. at 15 lb./in.^{2} pressure and sprayed with the modified Ehrlich reagent of Mauzerall & Granick (1956). Aminoketones give purple colours.

Growth of bacteria

Staphylococcus aureus (Duncan) was grown with constant shaking for 17 hr. at 37° in the 'deficient medium B' of Gale & Van Halteren (1952), except that the Marmite was replaced by Difco yeast extract. The cells were harvested and washed by suspension in 0·02M-phosphate buffer, pH 7·0, containing 0·1% of NaCl. For large-scale production of *S. aureus* the following procedure was adopted.

Batches of non-sterile medium (14 l.) were warmed to 37° in 20 l. flasks and each was inoculated with 1 l. of a fully grown culture of *S. aureus* obtained as above. Pre-warmed, water-saturated air was forced into the culture and growth allowed to continue for 6 hr. at 37°. The cells (from 30 l. of medium) were harvested on a Sharples centrifuge, washed by resuspension in 5 l. of water and again centrifuged. These cells were used in the experiments on the isolation of the new metabolite described later. Microscopic examination showed that the *S. aureus* preparations grown in this way were free from significant contamination by other micro-organisms.

Cell densities were measured in a Spekker absorptiometer with an Ilford 608 filter and dry weights were calculated from a calibration curve for this organism.

RESULTS

Formation of new substance from threonine

It was observed that when washed cell suspensions of *S. aureus* were incubated in buffered 0·9% NaCl in the presence of Difco yeast extract a small amount of an unidentified amino compound was seen on chromatograms of the incubation medium run in solvent I. The compound had R_F approx. 0·4. Increasing yields of the new substance were obtained as a result of increasing the yeast extract, up to a concentration of 0·1%, suggesting that the yeast extract was a source of substrate rather than of a cofactor. The component of the yeast extract responsible for this effect was found to reside entirely in the fraction absorbed by Dowex 50 (H^+ form), indicating that it was probably an amino acid, and this was confirmed by the finding that casein hydrolysate efficiently replaced the yeast extract. Each of 20 amino acids known to occur in casein was then tested for ability to promote synthesis of the new substance by washed suspensions of *S. aureus*, and, of these, threonine alone was found to be active.

Preliminary identification of the threonine metabolite

Washed cell suspensions of *S. aureus* were shaken for 3 hr. at 37° in the presence of 0·01M-L-threonine. The cells were removed by centrifuging and the supernatant was used for the preliminary determination of the properties of the metabolite.

Stability. The compound was apparently stable when solutions were heated in 6N-HCl at 105° for 16 hr. before chromatography. It was, however, completely destroyed when a solution was made alkaline with aq. NH_3 soln. or NaOH and shaken in air at room temperature for 6 hr. The compound was also destroyed when chromatographed in ammoniacal solvents.

Chemical reactions. A number of chromatograms were run and various sprays applied to the papers. The compound gave, with ninhydrin, an initial bright yellow which turned brown and finally deep purple at room temperature; it reacted with ninhydrin, after copper treatment, to give a permanent yellow, showing that it was not an α-amino acid; with ammoniacal $AgNO_3$ a reduction was obtained after 5 min. at room temperature. These properties suggested that the substance had an aminoketone structure, for such compounds undergo a Knorr type of condensation in alkaline solution and yield disubstituted pyrazines in the presence of air. This conclusion was confirmed by the finding that the material gave a positive reaction with the picrate spray and with the modified Elson–Morgan reaction when these were applied to chromatograms.

Chromatography. It was found that the new substance co-chromatographed with both of the two aminoketones, δ-aminolaevulinic acid and aminoacetone, when solvent I and IV were used. In solvent III, however, the substance ran ahead of δ-aminolaevulinic acid and had R_F identical with that of aminoacetone. A similar result was obtained with solvent II, when a good separation of δ-aminolaevulinic acid and aminoacetone occurred (R_F 0·15 and 0·3 respectively). These results suggested that the substance was in fact aminoacetone.

Isolation of aminoacetone

Experiments on the large-scale production of the compound were carried out so that the material could be isolated and positively identified.

The washed cells from 30 l. of growth medium were suspended at a concentration of 4 mg. dry w./ml. in 0·02 M-phosphate buffer, pH 7·0, at 37° and 0·25 vol. of 0·1 M-L-threonine, also at 37°, was added. The mixture (6 l.) was incubated in a 10 l. jar at 37° and aerated with water-saturated air dispersed by a sintered-glass diffusion disk. Samples of the mixture were removed at intervals for aminoacetone determination. Fig. 1 shows the time course of its production. The incubation was stopped after 17 hr., when production was virtually maximal with the formation of approx. 1 μmole/ml. of incubation medium. This amount corresponds to about 10 % of the L-threonine added.

Fractionation on Amberlite IRC 50. The suspension was adjusted to pH 5·0 with acetic acid and the cells were removed by centrifuging. The supernatant was passed at the rate of 8–12 ml./min. through a column (55 cm. × 6 cm.) of Amberlite IRC 50, buffered at pH 4·9 with M-sodium acetate–acetic acid buffer. The column was washed with water until the effluent gave a negative ninhydrin test. The basic fraction was eluted with 2 N-HCl until 1 ml. of the eluate gave a negative picrate reaction for aminoketone. Recovery of aminoacetone from the column was 80 %. At this stage the eluates from three similar preparations were combined.

Fractionation with phosphotungstic acid. The combined eluates were concentrated *in vacuo* until a moist solid, consisting mainly of NaCl, was obtained. This material was extracted with 400 ml. of absolute ethanol, filtered and the filter cake washed with ethanol. To the combined filtrate and washings (800 ml.) was added 100 ml. of water and the solution freed from ethanol by concentration *in vacuo* to about 20 ml. The concentrate was diluted to 400 ml. and 180 ml. of 10 % (w/v) purified phosphotungstic acid in 0·25 N-HCl solution was added. (The solution of aminoacetone was required to be 0·25–0·4 N with respect to HCl; a small sample was titrated and adjustments were made, if necessary, before phosphotungstic acid was added.) A voluminous precipitate formed and, after standing overnight at 0°, was removed by centrifuging. The supernatant was extracted with a mixture of amyl alcohol–ether (1:1, v/v) until free from phosphotungstic acid (Van Slyke *et al.* 1942).

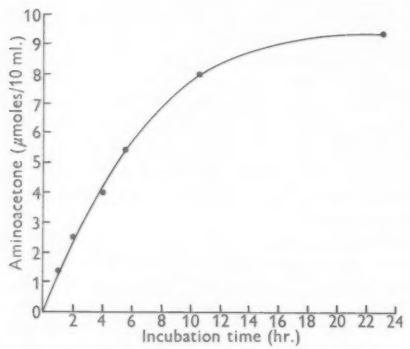

Fig. 1. Aminoacetone formation by washed *S. aureus* suspensions in large-scale experiment. A portion (6 l.) of washed *S. aureus* suspension (3·2 mg. dry wt./ml.) containing 20 mM-DL-threonine and 16 mM-phosphate buffer, pH 7·0, was incubated as described in the text. Samples were removed at intervals for aminoacetone determination.

Paper chromatography. The aqueous solution was concentrated to a syrup *in vacuo* to remove excess of HCl and diluted with 100 ml. of water. It was adjusted to pH 3·0–4·0 by the addition of N-NaOH and freeze-dried. The solid was extracted with 50 ml. of anhydrous ethanol and the insoluble NaCl filtered off. The filtrate was acidified by the addition of 0·1 ml. of 4 N-HCl and concentrated *in vacuo* to 15 ml., after which it was applied as continuous bands across 18 washed Whatman no. 3 papers (58 cm. wide). After development in butanol–acetic acid for 24 hr., the papers were dried at 37°. To locate the aminoacetone bands, use was made of the facts that the chromatograms always had a number of zones visible in u.v. light and that all bands on a given chromatogram had the same contours. The bands visible in u.v. were outlined in pencil and the aminoacetone band was located at one point by applying a narrow streak of ninhydrin down the paper and developing the colours at 30°. The aminoacetone band was then pencilled in, parallel to the other bands visible in u.v., and cut out. This procedure made possible accurate location of the bands even when they were irregular in shape. The aminoacetone was eluted chromatographically with 0·2 N-HCl and the solution concentrated *in vacuo* to a small volume of thick brown oil. This was dissolved in 6·5 ml. of anhydrous ethanol, left at −10° overnight and the resulting flocculent precipitate removed by centrifuging. Chromatography of the supernatant showed that only one ninhydrin-reacting component was present but there was also some u.v.-absorbing material.

*Crystallization as the toluene-*p*-sulphonic acid salt.* The clear-yellow supernatant (6 ml.) was mixed with an equal volume of ethanol containing 2 g. of toluene-*p*-sulphonic acid. Ether was added (approx. 1 ml.) until a dense precipitate formed. The mixture was left at 0°, after which the

precipitate was removed by centrifuging and discarded. More ether (about 15 ml.) was added to the supernatant until a faint turbidity appeared. Needle-shaped crystals formed after 5 min. at room temperature. The flask was left at 0° and the crystals were collected on a Büchner funnel and washed in ether–ethanol (9:1, v/v). A second crop of crystals was obtained by the addition of more ether and the two lots were combined. The crystals were dried over P_2O_5 *in vacuo*; yield, 0·39 g.

The product was recrystallized twice by dissolving it in ethanol and adding ether as before, and finally obtained as fine white needles, m.p. 130–131° (uncorr.) (Found: C, 49·2; H, 6·3; N, 5·9; S, 13·0. $C_{10}H_{15}O_4NS$ requires C, 49·0; H, 6·2; N, 5·7; S, 13·1 %).

Synthetic aminoacetone toluene-*p*-sulphonic acid salt was recrystallized twice and obtained as thin plates with m.p. 130–131° (uncorr.) (Found: C, 49·1; H, 6·4; N, 5·9; S, 12·9 %). The melting point was unchanged by admixture with the salt of the natural compound. The identity of the natural and synthetic salts was confirmed by infrared spectroscopy.

The isolated salt and the synthetic material had the same R_F when chromatograms were developed in solvents I and II. Ionophoretic examination at pH 5·0 showed that both the natural and synthetic compounds migrated towards the cathode at the same speed.

Formation of 2:4-dimethyl-3-acetylpyrrole. A portion (50 mg.) of the isolated salt was dissolved in 4 ml. of M-acetate buffer, pH 4·6, 0·1 ml. of acetylacetone was added and the solution heated at 100° for 15 min. The solution was cooled and, on adjustment to pH 8·0 with NaOH, crystals of 2:4-dimethyl-3-acetylpyrrole appeared. The crystalline material was filtered off, washed with a little ice-cold water and dried *in vacuo* over P_2O_5; m.p. 138–140° (corr.) (cf. 137°, Knorr & Lange, 1902). The m.p. was unchanged by admixture with the substituted pyrrole prepared in the same way from synthetic aminoacetone toluene-*p*-sulphonic acid salt.

The u.v. spectrum of the pyrrole formed from the natural aminoacetone salt had a maximum at 251 mμ, a shoulder at 275–286 mμ and a minimum at 227 mμ, in agreement with the data reported by Cookson (1953) for this compound.

The pyrroles from the salts of both the synthetic and natural aminoacetone gave immediate reactions with Ehrlich reagent and the coloured complexes derived from the two samples had identical absorption spectra.

Factors affecting aminoacetone formation from threonine

Table 1 shows that aminoacetone formation is dependent on the presence of unboiled *S. aureus* cells. None is formed if the cells are first boiled and a negligible amount is formed in the absence of threonine. When the incubation was carried out under anaerobic conditions the yield was decreased by 81 %.

Cell density. Fig. 2 shows that aminoacetone formation is proportional to cell density up to a value of 2 mg. dry wt./ml., after which some factor, possibly oxygen supply, becomes limiting.

Age of cells. No significant difference was observed in the rate of aminoacetone formation from threonine with cells harvested in the logarithmic phase of growth and those harvested at the end of the rapid-growth phase.

Table 1. *Aminoacetone formation from threonine by washed cell suspensions of* Staphylococcus aureus

The complete system contained 10 mM-phosphate buffer, pH 7·0, 5 mM-L-threonine and washed *S. aureus* suspensions (30 mg. dry wt./ml.); volume, 10 ml. Flasks were shaken at 37° for 3 hr. In experiment 4 the flask was evacuated and the air replaced with nitrogen.

Experiment	Aminoacetone: μmoles/10 ml.	Aminoacetone: Percentage of that in complete system
1 Complete system	8·15	—
2 Stopped at zero time	0·10	1
3 Cells boiled	0·30	4
4 Incubated under N_2	1·55	19
5 Threonine omitted	0·25	3

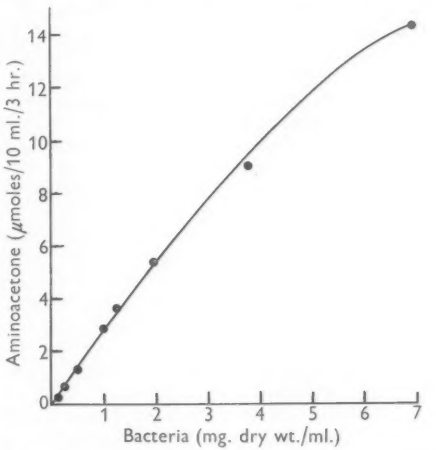

Fig. 2. Effect of cell density on aminoacetone formation from threonine. Washed *S. aureus* suspensions were incubated for 3 hr. at 37° with shaking in the presence of 40 mM-DL-threonine and 20 mM-phosphate buffer, pH 7·0.

pH. As might be expected with intact cells, there was a broad pH optimum centred around pH 7·1, the activity falling by only 20% at pH 5·2.

Threonine concentration. The system is saturated with L-threonine at 0·01 M. The apparent K_m is approx. 1·5 mM.

Specificity. No aminoketone formation could be detected by the picrate reaction when threonine was replaced by DL-*allo*threonine or by DL-β-hydroxyglutamic acid. It is possible, however, that these substances did not penetrate the cell wall of the organism. The yield from DL-homoserine, the structural isomer of threonine, was only 23% of that obtained from DL-threonine, but it remains to be determined whether this was due to homoserine alone or to contamination of it by threonine. L-Serine did not increase the yield of aminoacetone, nor did it give rise to the formation of a carbonyl–amino compound as judged by the picrate reaction, the modified Elson–Morgan reaction and by paper chromatography.

Effect of carbon dioxide. Aminoacetone formation from threonine must involve decarboxylation at some stage and it was therefore tested if there was any inhibition of its formation by CO_2. No such effect was observed. Flasks gassed with O_2 or with O_2+CO_2 (95:5) in the presence of 3·7 mM-$NaHCO_3$ gave identical yields.

Attempt to find a decarboxylation product of threonine

The initial attack on the threonine molecule by the *S. aureus* system might be expected to involve either an oxidation or a decarboxylation step. If the latter were the case it is possible that there would be some accumulation of 1-amino-2-propanol when aminoacetone formation was prevented by incubation of the system under anaerobic conditions. To test for this, experiments were carried out as follows. Incubation mixtures containing 20 mM-L-threonine were shaken with washed *S. aureus* suspensions at 37° for 3 hr., the gas phase in the flasks being air in one case and N_2 in the other. After incubation, the mixtures were acidified by the addition of 1·5 ml. of N-HCl and boiled for 2 min. to release cell contents. The supernatants, after centrifuging, were adjusted to pH 5·0 and the bases absorbed on to Amerlite IRC 50 columns. After washing, the columns were eluted with HCl, the eluates were evaporated to dryness *in vacuo* and the residues dissolved in water. The salts present were precipitated by the addition of 4 vol. of ethanol, removed by centrifuging and the supernatants were used for chromatography in solvent II.

It was found that in the aerobic experiment the major ninhydrin-reacting component present was aminoacetone. In the anaerobic experiment there was only a trace of aminoacetone and there was no evidence of any accumulation of an amino alcohol. Although a marker of 1-amino-2-propanol was not available, ethanolamine, the lower homologue, ran almost as fast as aminoacetone, and in both the anaerobic and aerobic experiments the only fast-moving component was aminoacetone itself. There was thus no detectable accumulation of a decarboxylation product of threonine under anaerobic conditions.

Aminoacetone synthesis from glycine

The preceding results show that aminoacetone is formed from threonine by *S. aureus*. Although the presence of glucose is not obligatory for this reaction, it was observed that when suspensions of the organism were incubated in the presence of

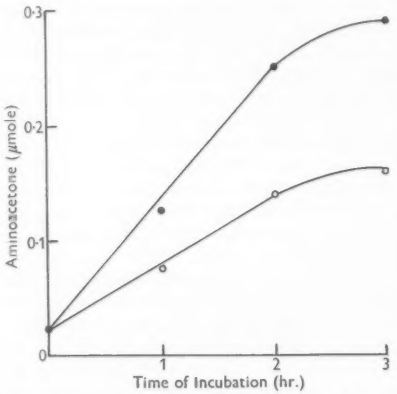

Fig. 3. Aminoacetone synthesis from glycine by washed *S. aureus* suspensions. Washed *S. aureus* (2 mg. dry wt./ml.) were incubated with shaking at 37° in the presence of 33 mM-phosphate buffer, pH 7·0, 10 mM-glycine and 1% glucose. Volume, 20 ml. ●, Complete system; ○, glycine omitted.

Table 2. *Synthesis of aminoacetone from glycine by washed* Staphylococcus aureus *suspensions*

Washed *S. aureus* suspensions (4 mg. dry wt./ml.) were incubated, with shaking, at 37° for 3 hr. in the presence of 33 mM-phosphate buffer, pH 7·0, 10 mM-glycine and 1% glucose. Volume, 20 ml.

Experiment	Aminoacetone μmoles/20 ml.	Aminoacetone Percentage of that in complete system
1 Complete system	1·23	—
2 Stopped at zero time	0·08	7
3 Glycine omitted	0·40	34
4 Glucose omitted	0·33	28
5 Complete, incubated under N_2	0·43	36

Table 3. *Retention of glycine metabolite on Amberlite IRC 50*

Experimental details are described in the text.

Experiment	Fraction	Aminoacetone	
		μmoles/10 ml.	Percentage of that in complete system
1 Complete system	Total aminoketone	0·46	—
2 Complete system	Non-basic aminoketone	0·02	4
3 Glucose omitted	Total aminoketone	0·13	28

glucose alone, a small amount of a compound displaying the properties of an aminoketone was produced. The chromatographic behaviour and chemical reactions of the product were identical with those of aminoacetone. The addition of glycine caused a stimulation of the rate of synthesis of the material which proceeded linearly for 2 hr. (Fig. 3), though the maximum rate was only about 6% of that with threonine.

Maximum formation of the metabolite from glycine is dependent on the presence of glucose (Table 2), and omission of this substrate diminished the yield by 72%. It is probable that sufficient oxidizable substrate is always present to permit some synthesis and it is noteworthy that *S. aureus* cells invariably contained some glycine. As with aminoacetone formation from threonine, O_2 is necessary for aminoketone formation from glycine. Replacement of air in the incubation flask by N_2 caused a 64% inhibition of aminoketone synthesis from glycine (Table 2). In this experiment no attempt was made to remove dissolved O_2 from the system.

Specificity. Glycine could not be replaced by alanine or by a mixture of alanine and ammonia. It can therefore be concluded that the glycine is not merely a source of amino groups or of ammonia. Glucose could not be replaced by sodium pyruvate, although this result may be due to a failure of the substrate to penetrate the cell.

Accumulation of aminoketone outside the cell. It was investigated whether or not the aminoketone was freely diffusible from the *S. aureus* cell. Incubations were carried out under the conditions described in Table 2, with glycine and glucose present. After incubation for 3 hr. in air the reaction in one flask was stopped by centrifuging the intact cells; the second flask was acidified by the addition of 0·4 ml. of 5N-HCl and the suspension heated to 100° for 1 min. to release the cell contents, cooled and centrifuged. It was found that amounts of aminoacetone in the two supernatants were 0·45 and 0·48 μmole respectively, showing that the metabolite was not concentrated to any significant extent within the bacterial cells.

Properties of the glycine metabolite

The glycine metabolite gave the same colour reactions on paper as did the threonine metabolite. Moreover, it did not separate from aminoacetone when chromatographed in any of the acidic solvents used in this work. Two-dimensional chromatography in solvents I and III showed that the glycine metabolite could be separated from δ-aminolaevulinic acid and moreover that the unfractionated eluates from Dowex 50 columns (Elliott, 1960) contained none of the latter compound. There was insufficient of the glycine metabolite formed to make its isolation feasible and, because of its low concentration relative to salts present in the incubation medium, it was not possible to determine the charge on the molecule by paper electrophoresis. It was therefore necessary to resort to ion-exchange resins to establish its basic nature.

Fractionation on Amberlite IRC 50. An incubation mixture (190 ml.) containing glycine and glucose as described in Table 2 was set up together with a control experiment in which glucose was lacking. After incubation the total aminoketone present in samples of both mixtures was estimated after Dowex 50 treatment (Elliott, 1960).

The remainder of the complete incubation mixture was adjusted to pH 5·0, the cells were removed and the supernatant was passed through a column (20 cm. × 2 cm.) of Amberlite IRC 50 buffered at pH 4·9 and washed through with water. The volume of the combined effluent and washings was measured and an appropriate sample taken for estimation of the non-basic aminoketone (i.e. that not retained by Amberlite IRC 50). Essentially all of the aminoketone was retained on the Amberlite resin (Table 3), showing that all of the aminoketone, whether dependent for its formation on glucose or not, is basic in nature. This rules out the possibility of there being formed a mixture of a basic and a non-basic component.

The basic compounds were eluted from the Amberlite columns with 2N-HCl and the eluent was evaporated to dryness *in vacuo*. The solids were extracted with ethanol, the solution was

evaporated to dryness once and extracted with 2 ml. of anhydrous ethanol. Chromatography in solvent II showed it to have the same R_F as aminoacetone.

DISCUSSION

The experimental work described shows that aminoacetone is produced by washed suspensions of *S. aureus* by two apparently distinct metabolic routes.

The conversion of threonine into aminoacetone presumably involves both the oxidation of the β-hydroxyl group and the loss of the carboxyl group, unless some more complicated pathway is postulated. Assuming the first step to be an oxidation, this would result in the formation of α-aminoacetoacetic acid (reaction 1), which would spontaneously break down to aminoacetone (reaction 2).

$$CH_3 \cdot CH(OH) \cdot CH(NH_2) \cdot CO_2H \xrightarrow{-2H} CH_3 \cdot CO \cdot CH(NH_2) \cdot CO_2H \quad (1)$$

$$CH_3 \cdot CO \cdot CH(NH_2) \cdot CO_2H \rightarrow CH_3 \cdot CO \cdot CH_2 \cdot NH_2 + CO_2 \quad (2)$$

Laver, Neuberger & Scott (1959) have shown that synthetic α-aminoacetoacetic acid, prepared from its ester, has a half-life of very much less than 1 min. at pH 7·0 and room temperature.

On the other hand, if decarboxylation of threonine occurred first, 1-amino-2-propanol would be formed (reaction 3) and a second enzyme would be required for its further conversion into aminoacetone (reaction 4).

$$CH_3 \cdot CH(OH) \cdot CH(NH_2) \cdot CO_2H \rightarrow CH_3 \cdot CH(OH) \cdot CH_2 \cdot NH_2 + CO_2 \quad (3)$$

$$CH_3 \cdot CH(OH) \cdot CH_2 \cdot NH_2 \xrightarrow{-2H} CH_3 \cdot CO \cdot CH_2 \cdot NH_2 \quad (4)$$

Krasna, Rosenblum & Sprinson (1957) found that the 1-amino-2-propanol moiety of vitamin B_{12} was derived from threonine and it was suggested that a decarboxylation of the amino acid takes place.

Experimentally it has been found that there is no detectable accumulation of 1-amino-2-propanol when aminoacetone formation from threonine is prevented by incubating the system under anaerobic conditions. This result, together with the fact that there is no lag period in the formation of aminoacetone under aerobic conditions, favours the view that aminoacetone arises from threonine via reactions 1 and 2. Studies of the enzyme system at the cell-free level are required before this can be determined with certainty, however.

Although the aminoketone derived from glycine has not been positively identified by isolation, its properties warrant the assumption that it is, in fact, aminoacetone. In considering the mechanism of its synthesis from glycine it should be pointed out that threonine is an essential amino acid for *S. aureus* (Gale & Folkes, 1953). It is therefore unlikely that aminoacetone formation from glycine occurs via threonine, though perhaps the possibility should not be entirely discounted. The requirements of the system responsible for the synthesis of aminoacetone from glycine are consistent with the mechanism shown in reaction 5. In this it is envisaged that acetyl-coenzyme A (CoA), derived from glucose, condenses with glycine to yield α-aminoacetoacetic acid.

$$CH_3 \cdot CO \cdot S \cdot CoA + CH_2 \cdot NH_2 \cdot CO_2H \rightarrow CH_3 \cdot CO \cdot CH(NH_2) \cdot CO_2H + CoA \cdot SH \quad (5)$$

Aminoacetone would arise as before by the spontaneous decarboxylation shown in reaction 2. This scheme is based on that proposed by Nemeth, Russel & Shemin (1957) for the synthesis of δ-aminolaevulic acid from succinyl-CoA and glycine. Since the present work was done, Gibson, Laver & Neuberger (1958) and Kikuchi, Kumar & Shemin (1959) have in fact demonstrated aminoacetone synthesis from acetyl-CoA and glycine by red-blood-cell preparations. The existence of such a reaction was foreshadowed by the earlier suggestion of Nemeth *et al.* (1957) that the succinyl-glycine reaction might also apply to an acetyl derivative.

The question arises whether aminoacetone is a normal metabolite in animals and bacteria. No systematic study of the distribution of the threonine system has yet been made, though it was found in the one case tested that *Streptococcus faecalis* produced an aminoketone from threonine, although more slowly than did *S. aureus*. The glycine system appears to occur both in animals and bacteria and particularly noteworthy is the observation by Gibson *et al.* (1958) that red-blood-cell preparations form aminoacetone when incubated with glycine and pyruvic acid. It is likely therefore that the compound is a physiologically occurring metabolite. Mauzerall & Granick (1956) found that, of the total pyrrole formed by condensation of acetylacetone with the aminoketones present in normal human urine, 20–40 % was of a type derived from an aminoketone lacking a carboxyl group. This observation has been confirmed by the finding (unpublished observations) that the basic fraction of human urine from normal adults gives rise to a pyrrole on condensation with acetylacetone, which is extractable into ether at pH 8·0. The material gave a coloured complex with Ehrlich reagent whose absorption spectrum was the same as that given by the pyrrole derived from aminoace one.

It has been suggested (Elliott, 1959) that the above facts may be integrated into a metabolic cycle in which it is postulated that aminoacetone is deaminated to yield methylglyoxal, which would then be rapidly converted into D-lactic acid by the widespread glyoxalase system. The presence of an enzyme system capable of oxidizing D-lactic acid to pyruvate has been shown to be present in liver and kidney preparations of rat (Huennekens, Mahler & Fordman, 1951; Mahler, Tomisek & Huennekens, 1953) and in bacteria (Kaufman, Korkes & Del Campillo, 1951). This system, together with pyruvate oxidase, would regenerate acetyl-CoA. It was reported previously by Nemeth *et al.* (1957) that [^{14}C]aminoacetone was converted into $^{14}CO_2$ and into purines by the rat, and these authors suggested that this aminoketone might be involved in the oxidative metabolism of glycine, but they also stated that there was no evidence that aminoacetone was produced by the cell. The postulated cycle would provide a pathway for the oxidation of threonine and glycine and in the latter case would be an alternative route to the succinate–glycine cycle (Shemin, 1955).

The accumulation of aminoacetone by *S. aureus* would occur if the organism were incapable of deaminating aminoacetone. Such a metabolic defect would be in accordance with the limited nitrogen metabolism implied by the strict amino acid requirements of the organism.

It remains to be established whether or not aminoacetone is metabolized by the pathway suggested and, if so, what the quantitative importance of the mechanisms might be.

SUMMARY

1. Washed suspensions of *Staphylococcus aureus* form an aminoketone when shaken in air in the presence of threonine.

2. The threonine metabolite has been isolated in crystalline form as the salt of toluene-*p*-sulphonic acid and has been positively identified as aminoacetone.

3. A compound with properties indistinguishable from those of aminoacetone is synthesized in *Staphylococcus aureus* by what is apparently a second route requiring the presence of glycine, glucose and oxygen.

4. The mechanisms involved in the formation of aminoacetone by the two routes are discussed.

5. The possible role of aminoacetone in metabolism is discussed. It is suggested that the metabolite may be oxidized by a metabolic cycle in which the compound is converted into methylglyoxal and thence to pyruvic acid via the glyoxalase and D-lactate dehydrogenase systems.

I wish to thank Professor A. H. Ennor for the advice and encouragement which he has given during this work. My thanks are also due to Dr W. G. Laver, who suggested the use of toluene-*p*-sulphonic acid for crystallizing aminoacetone, to Dr E. S. Spinner for carrying out the infrared spectroscopy and to Dr Joyce Fildes for elementary analyses. I am also grateful to Dr D. J. Brown for advice on isolation procedures.

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The Hydrolysis of Glucose Monophosphates by a Phosphatase Preparation from Pea Seeds

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Although many phosphatase preparations have been obtained from plant tissues there are few data available on the breakdown of glucose monophosphates, and of glucose 1-phosphate in particular. In the work of Porter (1953), Spencer (1954) and Roberts (1956), it was found that glucose 1-phosphate was hydrolysed by extracts from potato tubers, tomato leaves and wheat leaves respectively; however, more information is desirable on whether glucose 1-phosphate was hydrolysed directly, or through the intermediate formation of glucose 6-phosphate by phosphoglucomutase and the subsequent hydrolysis of the 6-ester. Axelrod (1947) found that glucose 1-phosphate was not hydrolysed by the acid phosphatase of citrus fruit although other phosphoric compounds such as fructose 1:6-diphosphate were hydrolysed.

With extracts from some animal tissues it has been shown that the hydrolysis of glucose 1-phosphate may take place via glucose 6-phosphate. Broh-Kahn & Mirsky (1948) could find no evidence for the existence of a glucose 1-phosphatase in liver extracts, and Goodlad & Mills (1957) concluded that the main route of glucose 1-phosphate hydrolysis in rat liver is through a preliminary conversion into glucose 6-phosphate. The direct hydrolysis of glucose 1-phosphate by a hexose 1-phosphatase from silkworm blood was reported by Faulkner (1955); this extract did not attack glucose 6-phosphate. Morton (1955) found that purified alkaline phosphatases from cow's milk and calf intestinal mucosa hydrolysed both glucose 1-phosphate and glucose 6-phosphate.

Previous investigations (Turner & Turner, 1957) had indicated the presence of glucose monophosphatase activity in extracts from pea seeds. When glucose 1-phosphate was incubated with these extracts, glucose and inorganic phosphate were formed. However, further work was needed to establish that this hydrolysis did not proceed by way of glucose 6-phosphate.

In the present investigation the acid phosphatase from pea seeds was purified 20-fold and a number of its properties studied. Both glucose 1-phosphate and glucose 6-phosphate were hydrolysed directly although the rate of hydrolysis of glucose 6-phosphate was higher than that of glucose 1-phosphate. Inorganic phosphate acted as a competitive inhibitor of the phosphatase action.

MATERIALS AND METHODS

Substrates

Glucose 1-phosphate (G 1-P) was prepared by an unpublished method of Professor C. S. Hanes and Dr R. Hill. Fructose 6-phosphate (Neuberg, Lustig & Rothenberg, 1943) and fructose 1:6-diphosphate (Neuberg & Lustig, 1942) were also prepared in the laboratory. Glucose 6-phosphate (G 6-P) and 3-phosphoglycerate were obtained from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A., galactose 1-phosphate and *p*-nitrophenyl phosphate from Sigma Chemical Co., St Louis, Mo., U.S.A., and adenosine monophosphate and adenosine triphosphate from Pabst Laboratories, Milwaukee, Wis., U.S.A.

Preparation of enzyme extracts

Pea-seed acid phosphatase. Immature pea seeds (*Pisum sativum* L., var. 99L) (40 g.) were blended with 40 ml. of glass-distilled water at 4°. The mixture was centrifuged at 1000 *g* for 10 min. and the supernatant centrifuged at 20 000 *g* for 20 min. at room temperature. Saturated $(NH_4)_2SO_4$, pH 6·0, was added to the clear supernatant at 4°, and the fraction precipitating between 55 and 75% saturation was suspended in 7 ml. of water and dialysed with rocking against glass-distilled water for 2·5 hr. at 4°.

The slightly turbid phosphatase extract could be stored at −15° for 2 months with little loss of activity. The preparation contained approx. 4·9 mg. of protein/ml. and was used for all the studies reported except for those on the substrate specificity.

Purified pea-seed acid phosphatase. The dialysed extract (pH 5·8) was brought to pH 3·6 by the addition of 0·1 N-HCl, centrifuged and the clear supernatant adjusted to pH 5·8 with 0·1 N-NaOH. This was then stirred with 0·1 vol. of calcium phosphate gel (Keilin & Hartree, 1938) containing 10 mg. of $Ca_3(PO_4)_2$/ml. at room temperature for 15 min. and centrifuged. The precipitate was stirred with 0·15 M-phosphate buffer (KH_2PO_4–NaOH), pH 7·0, for 15 min., centrifuged and the precipitate again stirred with phosphate buffer. The combined supernatants were dialysed against glass-distilled water for 3·5 hr. at 4°. The preparation contained 0·89 mg. of protein/ml. and was used for the study of the substrate specificity of the preparation.

The purification effected by these treatments is shown in Table 1. There was no significant change in the ratio of glucose 6-phosphatase activity to glucose 1-phosphatase

Table 1. *Purification of pea-seed acid phosphatase*

Reaction mixtures were of the composition described for the assay of phosphatase activity. The amount of enzyme fraction added was adjusted so that not more than 10% hydrolysis of the substrate occurred in the reaction time.

Fraction no.	Treatment	Total protein content of fraction (mg.)	Phosphatase activity (μm-moles of phosphate liberated/mg. of protein/min.) G 1-P	Phosphatase activity (μm-moles of phosphate liberated/mg. of protein/min.) G 6-P	Ratio: glucose 6-phosphatase / glucose 1-phosphatase
I	Crude extract dialysed	429	5·4	31·5	5·8
II	$(NH_4)_2SO_4$ precipitation, dialysis	40·3	52·6	324·0	6·2
III	Fraction II adjusted to pH 3·6, supernatant adjusted to pH 5·8	23·4	72·6	464·5	6·4
IV	Fraction III adsorbed and eluted from $Ca_3(PO_4)_2$ gel, dialysed	8·98	100·0	637·0	6·4

activity during the course of the purification procedure. The glucose 1-phosphatase was purified 18-fold and the glucose 6-phosphatase 20-fold.

Pea-seed phosphoglucomutase. A crude extract from dried pea seeds was prepared by the method of Turner (1957) and centrifuged at 20 000 *g* for 20 min. at room temperature. The supernatant was fractionated by addition of saturated $(NH_4)_2SO_4$, pH 7·0; the precipitate was collected between 55 and 80% saturation, dissolved in water and dialysed against 25 mM-phosphate buffer (KH_2PO_4–NaOH), pH 7·0, at 4° for 2·5 hr.

Enzyme digests

The reaction mixtures were maintained at 30°.

Acid phosphatase activity. For the assay of phosphatase, the enzyme (containing approx. 0·25 mg. of protein) was incubated with 2-amino-2-hydroxymethylpropane-1:3-diol (tris; 0·75 m-mole)–acetic acid buffer, pH 5·4, in a volume of 14·5 ml., and the reaction was started by the addition of 7·5 μmoles of substrate in 0·5 ml. of water. The reaction time was 60 min. with G 1-P as substrate and 10 min. with G 6-P. The amount of enzyme preparation added was adjusted to give not more than 10% hydrolysis of the substrate in these times; under these conditions the rate of reaction was approximately constant during the incubation period. For the study of the effect of various inhibitors, the enzyme was incubated in 14·5 ml. of buffer containing the inhibitor for 15 min. at 30°, and the reaction started by the addition of substrate.

In the determination of phosphatase activity the reaction mixtures were inactivated by the addition of 0·1 vol. of 40% (w/v) trichloroacetic acid, and samples were used for the estimation of inorganic phosphate. Centrifuging was not necessary except where crude extracts were used. In the study of phosphoglucomutase, 6 vol. of 6% (w/v) trichloroacetic acid was added to the reaction mixtures, which were centrifuged and samples taken for the estimation of acid-labile and inorganic phosphate. Trichloroacetic acid extracts of the reaction mixtures were kept at 0° and manipulations carried out without delay; under these conditions there was no detectable hydrolysis of G 1-P.

Analytical methods

Determination of inorganic orthophosphate. In preliminary studies and in some experiments with inhibitors, inorganic phosphate was estimated by the method of Allen (1940) as modified by Turner (1957). For other determinations, the method of Lowry & Lopez (1946) was used.

Acid-labile phosphate. This was determined by measuring the inorganic phosphate released in 7 min. in N-HCl at 100°, by the method of Allen (1940).

Detection of glucose. Glucose in the reaction mixtures was demonstrated on paper chromatograms by the methods of Turner, Turner & Lee (1957).

Determination of protein. The protein content of the enzyme preparations was estimated by the spectrophotometric method of Warburg & Christian (1941).

Table 2. *Phosphoglucomutase activity in extract from dried pea seeds*

Reaction mixtures contained 30 μmoles of G 1-P, tris (75 μmoles)–acetic acid buffer of required pH, $MgCl_2$ added as indicated and 0·5 ml. of pea-seed phosphoglucomutase preparation (containing 13·9 mg. of protein); total volume, 0·9 ml. Incubation time, 60 min. Temp. 30°.

pH	Final concn. of $MgCl_2$ (mM)	Decrease in acid-labile phosphate (μmoles/reaction mixture)
5·4	0	0·5
5·4	1	1·3
7·5	0	5·3
7·5	1	14·7

RESULTS

Some properties of the phosphoglucomutase from dried pea seeds

When G 1-P was incubated with the phosphoglucomutase preparation at pH 5·4 and 7·5 in reaction mixtures of the composition described in Table 2, in the presence and absence of soluble starch (final concn. 0·13%), there was no change in inorganic phosphate. This indicated that the preparation contained no starch phosphorylase and showed little phosphatase activity. It is probable

that starch phosphorylase was eliminated in the $(NH_4)_2SO_4$ fractionation and that phosphatase activity was suppressed by the high inorganic phosphate level in the reaction mixtures because of dialysis of the enzyme preparation against phosphate buffer.

Phosphoglucomutase activity, as measured by decrease in acid-labile phosphate, is shown in Table 2. The activity at pH 7·5 was over ten times that at pH 5·4. The addition of $MgCl_2$ (final concn. mM) increased the phosphoglucomutase activity approximately 2·5-fold.

Some properties of the phosphatase from pea seeds

The formation of inorganic phosphate from G 1-P and G 6-P on incubation with pea-seed phosphatase is shown in Fig. 1. G 6-P was hydrolysed at a considerably faster rate than G 1-P.

When trichloroacetic acid extracts of the reaction mixtures were chromatographed on paper, visual examination showed that glucose was the only free sugar present. There was no decrease in the sum of acid-labile phosphate plus inorganic phosphate during the hydrolysis of G 1-P, and there was no increase in inorganic phosphate in the presence of ammonium molybdate (mM) and starch (0·13 %). This concentration of molybdate completely inhibited phosphatase activity and thus would allow the detection of starch phosphorylase activity.

Effect of pH *on phosphatase activity.* The effect of pH on the hydrolysis of both G 1-P and G 6-P is shown in Fig. 2. The curves are similar, but G 6-P had optimum pH 5·4–5·7, whereas G 1-P had a more marked optimum at pH 5·4. With increase in pH the rate of reaction decreased rapidly until, at pH 7·6, the rate was negligible. The addition of $MgCl_2$ (final concn. mM) to the reaction mixtures at both pH 5·4 and 7·5 had no effect on the rate of hydrolysis of either G 1-P or G 6-P. There was no

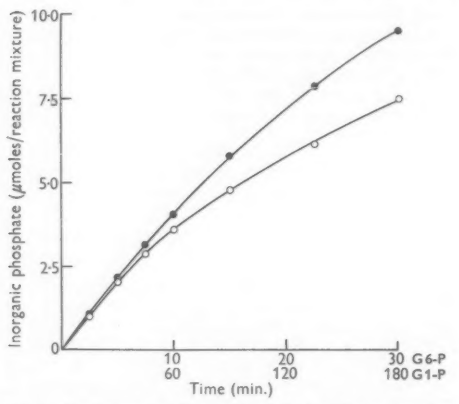

Fig. 1. Hydrolysis of G 1-P (○) and G 6-P (●) by pea-seed phosphatase. Reaction mixtures (total vol. 45 ml.) contained 22·5 μmoles of G 1-P (or G 6-P), tris (2·25 m-moles)–acetic acid buffer, pH 5·4, and 1·5 ml. of enzyme (containing 1·5 mg. of protein). Temp. 30°.

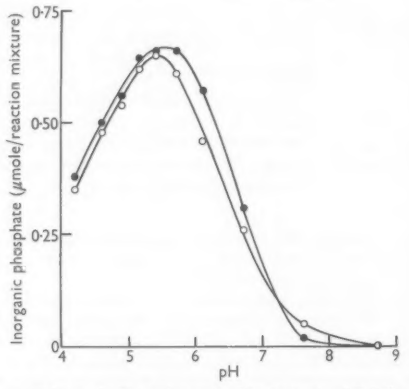

Fig. 2. Effect of pH on the rate of hydrolysis of G 1-P and G 6-P. Composition of the reaction mixtures was that described for the assay of phosphatase. Reactions were studied in tris (0·75 m-mole)–acetic acid buffers of pH indicated. Temp. 30°. ○, G 1-P, incubation time 60 min.; ●, G 6-P, incubation time 10 min.

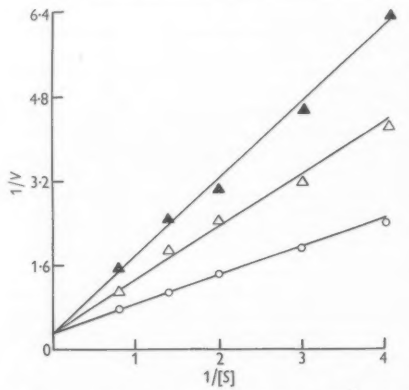

Fig. 3. Lineweaver–Burk plots [reciprocal of the initial rate of hydrolysis, V (μmoles of inorganic phosphate/reaction mixture), against the reciprocal of the substrate concentration, [S] (m-moles/l.)] for pea-seed phosphatase with G 1-P at two inorganic phosphate concentrations. The reaction mixtures (total vol. 15 ml.) contained G 1-P, tris (0·75 m-mole)–acetic acid buffer, pH 5·4, inorganic phosphate in concentrations given below and 0·5 ml. of enzyme (containing 0·25 mg. of protein). Temp. 30°. Incubation time 60 min. ○, No orthophosphate; △, 75 μM-KH_2PO_4; ▲, 150 μM-KH_2PO_4.

Table 3. *Effect of inhibitors on phosphatase activity*

Reaction mixtures were of the composition described for the assay of phosphatase with the addition of inhibitors as shown. Temp. 30°.

Inhibitor	Final concn. (mM)	Inhibition (%) Substrate G 1-P (incubation time 60 min.)	Inhibition (%) Substrate G 6-P (incubation time 10 min.)
Glucose	2·0	0	0
Inorganic phosphate	0·075	36	19
Inorganic phosphate	0·15	52	29
Iodoacetate	10·0	69	75
$HgCl_2$	0·2	74	73
Fluoride	2·0	75	78
Fluoride	20·0	100	100
Molybdate	1·0	100	100

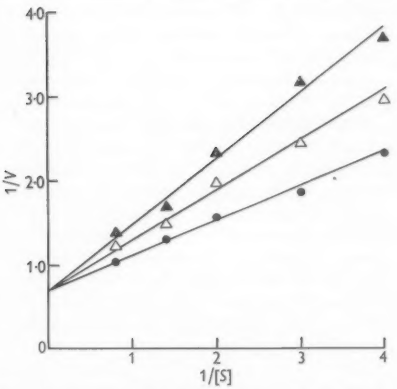

Fig. 4. Lineweaver–Burk plots [reciprocal of the initial rate of hydrolysis, V (μmoles of inorganic phosphate/reaction mixture), against the reciprocal of the substrate concentration, [S] (m-moles/l.)] for pea-seed phosphatase with G 6-P at two inorganic phosphate concentrations. The reaction mixtures (total vol. 15 ml.) contained G 6-P, tris (0·75 m-mole)–acetic acid buffer, pH 5·4, inorganic phosphate in concentrations given below and 0·5 ml. of enzyme (containing 0·038 mg. of protein). Temp. 30°. Incubation time 60 min. ●, No orthophosphate; △, 75 μM-KH_2PO_4; ▲, 150 μM-KH_2PO_4.

detectable decrease in the sum of acid-labile phosphate plus inorganic phosphate in any of the reaction mixtures.

Effect of concentration of substrates. The effect of concentration of G 1-P and G 6-P on the initial reaction velocity is shown in Figs. 3 and 4. The relative rate of hydrolysis of G 1-P to that of G 6-P increased with increasing substrate concentration.

Inhibition of phosphatase by inorganic phosphate. Preliminary experiments showed that the rate of reaction fell markedly as the reaction proceeded. Indications of this may be seen in Fig. 1, and experiments in which the reaction was allowed to proceed for longer periods suggested that the decline in rate was not predominantly due to substrate depletion. These observations indicated that products of the reaction may have been inhibiting phosphatase activity. The effects of addition of glucose and inorganic phosphate to the reaction mixtures are shown in Table 3. Glucose had no effect on phosphatase action whereas inorganic phosphate in low concentration inhibited the breakdown of both G 1-P and G 6-P.

The effects of two concentrations of inorganic phosphate on the rates of reaction with varying concentrations of G 1-P and G 6-P were examined, and in Figs. 3 and 4 the data are plotted according to the method of Lineweaver & Burk (1934). Inorganic phosphate was a competitive inhibitor of both glucose 1-phosphatase and glucose 6-phosphatase activities.

Effect of other inhibitors on phosphatase activity. The effects of addition of several other inhibitors are also shown in Table 3. Molybdate, iodoacetate, $HgCl_2$ and fluoride all inhibited the phosphatase activity. In separate experiments it was found that ethylenediaminetetra-acetate (final concn.

Table 4. *Hydrolysis of various substrates by purified pea-seed phosphatase*

Reaction mixtures contained 7·5 μmoles of substrate, tris (0·75 m-mole)–acetic acid buffer, pH 5·4, and purified pea-seed phosphatase (containing 0·06 mg. of protein); total volume, 15 ml. Incubation time was from 3 to 120 min., so that not more than 10% of substrate was hydrolysed. Temp. 30°.

Substrate	Relative rate of hydrolysis
G 1-P	1
G 6-P	6
Galactose 1-phosphate	1
Fructose 6-phosphate	8
p-Nitrophenyl phosphate	38
3-Phosphoglycerate	32
Adenosine monophosphate	2
Adenosine triphosphate	35
Fructose 1:6-diphosphate	15

10 mM) stimulated the phosphatase activity by approx. 20%.

Specificity of the phosphatase preparation. The action of the purified pea-seed phosphatase preparation on a number of phosphoric compounds is shown in Table 4. The enzyme preparation attacked a wide range of substrates and the hydrolysis of adenosine triphosphate, *p*-nitrophenyl phosphate, 3-phosphoglycerate and fructose 1:6-diphosphate was more rapid than that of the glucose monophosphates.

DISCUSSION

The present investigation has shown that both G 1-P and G 6-P are hydrolysed directly by the pea-seed phosphatase preparation. The lack of accumulation of acid-stable phosphate during the hydrolysis of G 1-P indicated that, if hydrolysis took place through G 6-P, phosphoglucomutase rather than phosphatase must be the rate-limiting step. The observations that the optimum pH for the hydrolysis of G 1-P (like that for G 6-P) was 5·4 whereas the activity of pea-seed phosphoglucomutase at pH 7·5 was approximately ten times that at pH 5·4 eliminated the possibility of hydrolysis via G 6-P. Additional evidence was provided by the addition of magnesium chloride, which markedly stimulated the action of pea-seed phosphoglucomutase but had no effect on the phosphatase. Cardini (1952) found that the phosphoglucomutase of the jack-bean had an optimum pH of 7·5 and also required Mg^{2+} ions. There was no inhibition of phosphatase activity by ethylenediaminetetra-acetate.

It is not known if two separate enzymes were responsible for the hydrolysis of G 1-P and G 6-P. There was no indication of any separation of the two activities during the purification procedures. The effects of various inhibitors on the hydrolysis of the two glucose monophosphates by the pea-seed preparations were similar.

Inorganic orthophosphate was a competitive inhibitor for the hydrolysis of G 1-P and G 6-P by the pea-seed phosphatase. An inhibition of this type may have been partly responsible for observations of low phosphatase activity in the presence of high starch-phosphorylase activity (Porter, 1953; Turner & Turner, 1957). Inhibition of phosphatase action by inorganic phosphate was observed by Jacobsen (1932, 1933), who concluded that the rate of hydrolysis of glycerophosphate by alkaline kidney phosphatase depended on the ratio of substrate to inorganic phosphate. Pfankuch (1936) found that the hydrolysis of α- and β-glycerophosphate, fructose 1:6-diphosphate, phosphoglycerate and phytate by potato phosphatase was inhibited by inorganic phosphate. Inhibition of calf intestinal mucosa alkaline phosphatase by disodium phosphate was observed by Morton (1955).

It is possible that inhibition of phosphatase by inorganic phosphate may have some metabolic significance because of the important role of inorganic phosphate in a number of processes. It has been suggested that a lowered concentration of inorganic phosphate may cause the inhibition of glycolysis by oxygen through the glyceraldehyde 3-phosphate dehydrogenase reaction (Johnson, 1941; Lynen, 1941); Lardy & Wellman (1952) have shown that inorganic phosphate may limit the rate of oxidation of respiratory substrates by mitochondria. If the concentration of inorganic phosphate fell to a low level (which might, for example, inhibit glycolysis) there would be a tendency for the glucose monophosphates (and possibly the other phosphoric compounds) to be hydrolysed and so increase the inorganic phosphate concentration. Conversely, if the inorganic phosphate concentration were increased (through anaerobiosis for example) there may tend to be a sparing effect on the phosphoric compounds due to inhibition of phosphatase action by the higher concentration of inorganic phosphate. The phosphatase may exert a balancing effect and so tend to stabilize the concentration of inorganic phosphate. An effect such as this would be analogous to the 'primitive' control mechanisms (i.e. mechanisms not controlled by hormones or a central nervous system) described by Krebs (1957).

SUMMARY

1. A phosphatase from pea seeds that hydrolysed both glucose 1-phosphate and glucose 6-phosphate was purified approximately 20-fold.

2. Glucose 1-phosphate was hydrolysed directly and not by way of glucose 6-phosphate.

3. The effects of pH, substrate concentration and inhibitors on the hydrolysis of the glucose monophosphates were studied.

4. The hydrolysis of other phosphoric compounds by the enzyme preparation was examined.

5. Low concentrations of inorganic phosphate competitively inhibited the hydrolysis of both glucose 1-phosphate and glucose 6-phosphate. The possible metabolic significance of this inhibition has been discussed.

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The Preparation of 2-Guanidinoethyl Phosphate

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During the isolation of lombricine from *Lumbricus terrestris* L. (Pant, 1959), it was observed that when the concentrated ammoniacal eluate from the C_{50}D cation column was kept in the refrigerator for 4–5 days, a white crystalline solid separated out. This compound on being purified was identified chromatographically as guanidinoethyl phosphate, which is one of the products of hydrolysis of lombricine (Van Thoai & Robin, 1954). Our repeated attempts to prepare this compound by the only available method described by Van Thoai & Robin (1954) proved unsuccessful. However, the recent synthesis of monophosphotaurocyamine by Morrison, Ennor & Griffiths (1958) suggested to us the possibility of its synthesis by a similar procedure.

This paper deals with the details of the preparation of guanidinoethyl phosphate and the study of some of its properties.

EXPERIMENTAL

Paper chromatography. Paper partition chromatography of Consden, Gordon & Martin (1944) was employed. Whatman no. 1 filter-paper sheets were used. The spots were developed at room temperature (22°) for about 7–8 hr. and the solvent was allowed to ascend a distance of about 23–25 cm. The operation was conducted in an air-tight glass-aquarium tank fitted with a lid. The atmosphere of the tank was kept saturated with the vapours of the solvent.

Spray reagents. The following were used: ninhydrin solution (0·1%, w/v) in butanol; Sakaguchi reagents (Pant, 1959) and molybdate reagents (Hanes & Isherwood, 1949).

Preparation of 2-aminoethanol 1-phosphate. This ester was prepared by treating phosphoryl chloride with aq. 2-aminoethanol as described by Outhouse (1937). To phosphoryl chloride (15 ml.) placed in a 500 ml. (Büchner) flask and cooled by immersion in a salt–ice mixture (−5°) an ice-cold mixture of 2-aminoethanol (10 ml., 1 mol.) and water (5 ml., 2 mol.) was added dropwise from a burette. The reaction proceeded with evolution of heat and copious fumes of HCl. When the reaction was complete, the flask was removed from the cold bath and evacuated with a water suction pump at room temperature for about 4 hr. until almost all the HCl formed had been removed. Water (500 ml.) was then added to the reaction mixture with stirring until the gummy mass formed was completely dissolved. The solution was made alkaline to pH 10·0 by addition of hot saturated baryta solution (approx. 400 ml.). The precipitated barium phosphate was centrifuged, washed twice with water (50 ml.), the washings were combined with the first supernatant and the volume was reduced to 50 ml. on a water bath. The solution was cooled and ethanol (80 ml.) was added in small quantities to precipitate the barium 2-aminoethanol 1-phosphate; it was then left at 0° for 2–3 hr. The white crystalline solid (8 g.) was centrifuged off and twice washed with ethanol (30 ml.) and ether and dried. The dry barium salt was recrystallized from water with ethanol and dried *in vacuo*. Yield, 7·5 g.

In order to obtain 2-aminoethanol 1-phosphate, the barium salt was dissolved in water (80 ml.) and H_2SO_4 (60 ml., 0·5N) was added to it until all the barium was completely precipitated as barium sulphate. The precipitate was centrifuged off, twice washed with water (25 ml.), the washings were combined with the original supernatant and the solution was concentrated on a water bath to 50 ml. The concentrate was filtered, made turbid by adding methanol (45 ml.) and left in the refrigerator overnight.

The crystals were filtered off, washed with methanol and ether and dried *in vacuo*. Yield, 4·5 g.; m.p. 243–244° uncorr. [Outhouse (1937), m.p. 244°; Clarke, Datta & Rabin (1955), m.p. (corr.) 243·5–244·5°; 243·0–244°]. The material on analysis gave P, 21·97; N, 9·81. $C_2H_8O_4NP$ requires N, 9·93; P, 21·99%. Chromatography in butanol–acetic acid–water (40:10:50, by vol.) gave only one ninhydrin-positive spot (R_F 0·13).

Guanylation of 2-aminoethanol 1-phosphate and synthesis of 2-guanidinoethyl phosphate. 2-Aminoethanol 1-phosphate was guanylated by *S*-methyl*iso*thiourea in an alkaline medium as described by Schütte (1943) for the preparation of guanidines. Thus 2-aminoethanol 1-phosphate (2 g.) was taken up in aq. NH_3 soln. (sp.gr. 0·88) in a 250 ml. flask and powdered *S*-methyl*iso*thiourea (2 g.) was added to it in small quantities at intervals of 30 min. with vigorous shaking. The reactants went into solution with the evolution of methanethiol. The reaction mixture was left at room temperature for 24 hr. in a fume cupboard and then distilled *in vacuo*. The syrupy mass left in the distillation flask was taken up in water (5 ml.) and filtered. Ethanol (20 ml.) was added to precipitate the guanylated derivative formed and the solution was kept at 0° for 2–3 hr. The white crystalline material was filtered off, washed with ethanol and ether and dried. The guanidine derivative was then purified by repeated crystallization with methanol from water and dried *in vacuo* over P_2O_5. Yield, 3 g. The m.p. was very high, the material remaining solid up to 290°.

The compound obtained gave a single spot on the paper chromatogram in all the solvents tested (1–8, Table 1) and did not reveal any ninhydrin-reacting spot. The spot gave a positive reaction with Sakaguchi reagents and showed the presence of phosphate on spraying with the molybdate reagents.

On hydrolysis with H_2SO_4 (6N, 100–110°) the compound gave guanidinoethanol and inorganic phosphate, as shown by paper chromatography. The compound, when run on a chromatogram together with a sample of hydrolysed lobmricine solution, revealed a spot on spraying with the Sakaguchi reagents, which coincided with the position of guanidinoethyl phosphate of lombricine hydrolysate and gave the same R_F, thereby indicating its identity.

On analysis the material gave C, 19·56; H, 5·52; N, 22·86; P, 16·94. $C_3H_{10}O_4N_3P$ requires C, 19·67; H, 5·46; N, 22·95; P, 17·21%.

Table 1. *R_F values of 2-guanidinoethyl phosphate developed with different solvent systems*

Except for solvent 1, the proportions of the components are by volume. In solvent 1, the atmosphere of the chromatographic tank was kept saturated by means of a beaker containing aq. NH_3 soln. (sp.gr. 0·88).

	Composition of solvent	R_F
1	Phenol–NH_3 (80% (w/v); NH_3 atm.)	0·47
2	Butanol–acetic acid–water (40:10:50)	0·13
3	Butanol–water–acetic acid (73:17:10)	0·05
4	Pyridine–*iso*pentanol–water–acetic acid (80:40:40:10)	0·19
5	Pyridine–*iso*pentanol–water (80:40:70)	0·22
6	Pyridine–*iso*pentanol–water–aq. 20% (v/v) NH_3 soln. (80:40:40:10)	0·05
7	Phenol saturated with water	0·49
8	Methanol–water–acetic acid (80:20:10)	0·51

One of us (Pant, 1959) has been able to phosphorylate this compound enzymically by employing an acetone-dried powder of *Lumbricus terrestris* L., as well as by using an homogenate of the worms in the presence of adenosine triphosphate.

DISCUSSION

The search for a method to prepare 2-guanidinoethyl phosphate led us to the only method previously described (Van Thoai & Robin, 1954). According to these authors, a suspension of guanidinoethanol in dry pyridine is added in small quantities to phosphoryl oxychloride in dry pyridine kept mechanically stirred and maintained at −5°. Calcium chloride is then added to the reaction mixture followed by calcium oxide to bring the pH to 8–9. The calcium phosphate is reported to precipitate and from the filtrate the guanidyl phosphate ester is isolated by precipitating with ethanol; the calcium is later removed as calcium oxalate by the addition of oxalic acid. However, the present workers, on repeating the above exactly and using the same quantities, encountered certain difficulties. In the first place, although the reaction mixture was stirred for over 2 hr. the guanidinoethanol did not go into solution. Secondly, it was not possible to adjust the pH of the non aqueous pyridine medium to 8·0 or 9·0. On the other hand, the synthesis of guanidinoethyl phosphate can easily be achieved by the new method described here. The operations involved are simple and the yield obtained is 75% of the theoretical.

SUMMARY

1. A new method has been described for the preparation of 2-guanidinoethyl phosphate by guanylation of 2-aminoethanol phosphate by *S*-methyl*iso*thiourea. Some of the properties of this compound have been reported.

Grateful acknowledgement is made to the Council of Scientific and Industrial Research for financing the scheme under which this work was carried out and for a research assistantship to one of us (S.S.D.).

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A Protein from Leaves Catalysing the Reduction of Haem–Protein Compounds by Illuminated Chloroplasts

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It was shown previously (Davenport, Hill & Whatley, 1952) that extracts of leaves contain a factor capable of catalysing the reduction of methaemoglobin and metmyoglobin by illuminated chloroplasts. Although this hydrogen-transferring capacity appeared to be analogous to that of ferric oxalate in a similar system (Hill & Scarisbrick, 1940), the naturally occurring substance was shown to be thermolabile, non-diffusible and stable only between the limits pH 5·0–9·0. Attempts at purification by fractional precipitation with ammonium sulphate gave active precipitates at near-saturation with the salt. It was concluded that the methaemoglobin-reducing activity was associated with a protein fraction of the leaf. In the earlier investigation it was found that the preparations of the factor were too unstable to allow a purification sufficient for characterization. It has now been found that if the leaves are held in a frozen state before extraction, the stability of the preparations was considerably improved and a highly purified product could be obtained. More recently (Hill, Northcote & Davenport, 1953) a similar activity was detected in cell-free extracts of the alga *Chlorella*. The present paper is concerned with the further purification of the 'methaemoglobin-reducing factor' and some additional properties are described. A brief account of this work was published previously (Davenport & Hill, 1955).

EXPERIMENTAL

Plant materials. Most of the plant material used in the preparation of leaf-protein fractions and of isolated chloroplasts was grown under ordinary garden conditions near Cambridge. Some species, however, notably *Lamium album*, were collected from their natural habitat as they became seasonally available.

Metmyoglobin. Crystalline whale metmyoglobin was prepared from sperm-whale muscle (obtained with the kind co-operation of Dr J. G. Sharpe, Low Temperature Research Station, Cambridge) by a method similar to that of Keilin & Schmidt (1948). The crystals were stored in 3 M-ammonium sulphate solution, and, for use, the ammonium sulphate was removed by dialysis against distilled water. The concentration of metmyoglobin was then estimated as cyan-metmyoglobin. ϵ_M^{540} was taken as $1{\cdot}11 \times 10^4$ and the stock solution was adjusted to 2 mM.

Cytochrome c. Horse-heart cytochrome *c* was prepared by the method of Keilin & Hartree (1945) with the additional purification step using the ion-exchange resin Amberlite IRC 50 (Rohm and Haas Co.) as advocated by Margoliash (1952). In the reduced state ϵ_M^{550} was assumed to be $2{\cdot}8 \times 10^4$.

Isolated chloroplasts. Chloroplasts were prepared from *Pisum sativum*, *Hordeum vulgare*, *Chenopodium bonus-henricus* and *Stellaria media* according to seasonal availability. Leaves were ground in a chilled mortar with about five times their weight of 6% glucose in 0·03 M-phosphate buffer, pH 7·4 (Sörensen, 1912). After coarse filtration through glass wool the larger particles were removed by centrifuging at 500 *g* and 0° for 2 min. The supernatant suspension was then recentrifuged at 2000 *g* for 10 min. to give a pellet consisting essentially of whole chloroplasts. This was rinsed with and resuspended in the cold glucose solution.

Chlorophyll concentration. This was estimated by the method of Mackinney (1941).

Boundary electrophoresis. For analytical purposes a Perkin–Elmer version of the Tiselius apparatus with a cell of 2 ml. capacity was used. Preparative use was also made of a large Tiselius apparatus of cell capacity 10 ml.

Paper electrophoresis. The method was essentially that of Durrum (1950). To minimize pH changes during a run large electrode vessels of capacity 2·5 l. were used. Phosphate buffers (0·066 M; Sörensen, 1912) containing mM-sodium ethylenediaminetetra-acetate proved most satisfactory. Whatman no. 3 papers up to 40 cm. total width could be accommodated. Direct current from a 200 v line was connected to platinum-foil electrodes to give a potential gradient on the paper of 5 v/cm. The apparatus was installed in a cold room at 4° and was enclosed in a glass container to reduce evaporation from the paper. At the end of a run, usually 5 hr., the coloured zones were cut out and the protein was eluted by downward capillary displacement.

Diffusion coefficient. For this measurement the Perkin–Elmer Tiselius apparatus was used. The formed boundary was displaced into the right limb of the cell by lowering a glass plunger, operated by clockwork, into the opposite electrode vessel. The schlieren pattern of the boundary was photographed at 1 hr. intervals for 6 hr.

Sedimentation coefficient. The observations were made with a Spinco analytical untracentrifuge at the maximum rotor speed of 59 780 rev./min. The schlieren pattern was photographed at intervals of 32 min. during the run.

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Chloroplast reaction-velocity measurements. When metmyoglobin is reduced in solutions saturated with air the myoglobin produced is immediately oxygenated to oxymyoglobin. The progress of the reaction was followed spectroscopically by the method previously described (Hill, 1936; Davenport *et al.* 1952). This method was also applied to the measurement of cytochrome *c* reduction, and then reduced and oxidized cytochrome replaced oxymyoglobin and metmyoglobin in the cups of the spectrocolorimeter.

Protein nitrogen. This was determined by the Kjeldahl procedure of Chibnall, Rees & Williams (1943).

RESULTS

Purification of the active leaf protein

The purification procedure to be described has been applied to leaves of *Pisum sativum*, *Lamium album*, *Chenopodium bonus-henricus*, *Aethusa cynapium* and *Stellaria media*. The choice of material was determined not only by the ultimate yield of active material but also by the absence of the tendency, common to plants rich in polyphenol oxidase, towards the production of dark pigments. These pigments may become firmly attached to the various protein fractions. In these respects *Pisum* proved to be the most satisfactory plant and the following method refers specifically to leaves from the garden pea.

Freshly picked leaves (2 kg.) were washed in tap water, drained by manual centrifuging in a muslin bag and rapidly chilled to $-20°$. They were held at this temperature for at least 2 days. When this freezing step was omitted rapid loss of activity occurred during subsequent fractionation. No explanation of this stabilizing effect can yet be suggested.

The frozen leaves were pounded in batches in a large mortar chilled to $-20°$ and accumulated as a frozen leaf powder. To the powder 1700 ml. of saturated ammonium sulphate solution was added with stirring to give a final concentration of the salt around 50% saturation. Stirring was then continued until the preparation thawed ($-3°$). It was then squeezed through two layers of muslin and the fibre discarded. To the press juice a further 1700 ml. of saturated ammonium sulphate solution was then added with stirring and the precipitate was removed by filtration on a Büchner funnel (18·5 c.) with Whatman no. 1 paper and 25 g. of Hyflo Supercel as filter aid. The filter pad was rejected and the filtrate fully saturated with ammonium sulphate by addition of the solid at room temperature. It is of importance at this stage to approach complete saturation as nearly as possible since the small precipitate contains the active material. This precipitate was removed by filtration as before but with 25 g. of Celite analytical-grade filter aid (Johns–Manville). Coarser grades of Celite do not retain the precipitate. The filtrate was rejected and the filter pad eluted with 0·06 M-phosphate, pH 7·0, to give 200 ml. of a brown extract. This extract was fractionated three times with ammonium sulphate. At each stage the precipitate between 80 and 100% saturation was removed by filtration and retained. The final filter pad was extracted with and dialysed against 0·01 M-phosphate, pH 7·0. The contents of the dialysis sac, containing 62 mg. of protein, were freeze-dried and stored over P_2O_5, where the activity is retained for at least 1 month.

Samples of the freeze-dried material were subjected to moving-boundary electrophoresis as described. At pH 7·2 (I 0·2) the boundaries of three components could be detected moving towards the anode. Although it was possible to establish that the most mobile component possessed metmyoglobin-reducing activity and that the least mobile component was inactive, the relative mobilities of the three components were too close for complete separation to occur. Since the three protein fractions were all acidic, attention was turned to electrophoresis on paper where the endosmotic flow acting in the opposite direction to electrophoretic migration might enhance the separation. Paper electrophoresis was carried out as described. The freeze-dried preparation was dissolved in a minimum volume of water and streaked on the paper at right angles to the machine direction at the rate of 1 mg./cm. width. At pH 7·2, after the current had passed for 5 hr., complete separation into three coloured zones occurred (Fig. 1).

On the anode side of the point of origin a pink zone (A) was clearly demarcated from a less-mobile brown zone (B). A yellow zone (C) appeared to migrate towards the cathode but by the use of glucose as an uncharged marker this effect was shown to be due to endosmotic flow. The protein nature of the coloured zones and the absence of

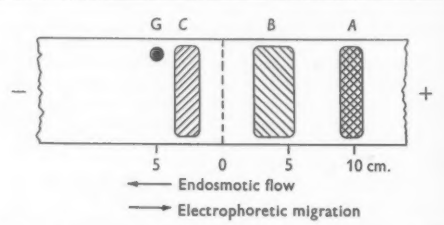

Fig. 1. Diagrammatic representation of the location of coloured protein zones on Whatman no. 3 paper strip after electrophoresis, relative to an uncharged marker (G, glucose). Pea-leaf protein applied at 0 cm. at 1 mg./cm. width; 0·06 M-phosphate, pH 7·2, containing 5 mM-sodium ethylenediaminetetra-acetate; 5 v/cm.; current on 5 hr.

uncoloured protein fractions was confirmed by dyeing a longitudinal strip of the paper in zinc sulphate–acetic acid–bromophenol blue reagent (Jencks, Jetton & Durrum, 1955).

After elution of the coloured zones from the paper it was confirmed that the most mobile component (*A*) was highly active in catalysing the reduction of metmyoglobin in presence of illuminated chloroplasts. Activity was absent from components *B* and *C* but spectroscopic examination of *C* after reduction with $Na_2S_2O_4$ revealed absorption bands at 559 and 530 mμ indicating the presence of cytochrome b_3 (Hill & Scarisbrick, 1951) in this fraction. The protein material from zones *A* and *C* was dialysed against 0·01 M-phosphate, pH 7·0, and accumulated in the freeze-dried state. From 2 kg. of pea leaves 16·6 mg. of protein was recovered in fraction *A*.

Properties of the purified protein

Sedimentation and diffusion measurements. Fraction *A* protein from pea leaves was examined in a Spinco analytical ultracentrifuge. At the maximum rotor speed of 59 780 rev./min. sedimentation was slow but only one symmetrical boundary could be detected. At pH 7·2 in phosphate buffer (*I* 0·2), $S_{20,w}$ was $1{\cdot}6 \times 10^{-13}$.

The diffusion coefficient was measured on the same sample of protein by the method described above. In phosphate–NaCl (Miller & Golder, 1950) at pH 7·0 (*I* 0·2), $D_{20,w}$ was $7{\cdot}72 \times 10^{-7}$.

From these values, and assuming a partial specific volume of 0·74, the calculated molecular weight ($M_{calc.}$) is 19 000.

Activity measurements. It was shown previously (Davenport *et al.* 1952) that the rate of reduction of metmyoglobin in a test system containing isolated chloroplasts, extracts of acetone-dried leaf and metmyoglobin is dependent upon the concentration of chlorophyll and metmyoglobin, the amount of added extract, the pH of the medium and the light intensity. These variables may be standardized but a further variable is the photochemical activity of the chloroplast preparations used. This activity, as measured by the ability of the preparation to reduce 2:6-dichlorophenolindophenol under standard conditions, may show wide variations from one preparation to another. Moreover, chloroplast preparations may lose activity rapidly after extraction (Hill, 1939). It was not therefore possible to devise a completely reproducible test system for the assay of activity of protein fractions isolated from the leaves. A compromise was reached by removing samples at different stages of a preparation and comparing their activity together, with pea chloroplasts where possible. These usually retain their activity unchanged for several hours at 0°.

The test mixture usually contained 0·04 mg. of chlorophyll, 0·4 μmole of metmyoglobin, the appropriate amount of leaf extract and 0·03 M-phosphate to 2·5 ml. The reaction was followed as described and the reaction rate expressed in terms of chlorophyll. $Q_{O_2}^{chl.}$ is defined as μl. of oxygen/mg. of chlorophyll/hr., where the volume of oxygen is the calculated stoicheiometric equivalent to the metmyoglobin reduced.

The earlier observations (Davenport *et al.* 1952), with crude leaf extracts, showed that progressive increments in the amount of leaf extract added led to increased reduction rates until the chloroplast system became saturated. Further additions then gave no further increase in reaction rate. Early in the present work it became apparent that purification of the crude extracts resulted in an increase in the $Q_{O_2}^{chl.}$ at saturation. This is illustrated in Fig. 2, where the rate curve for a crude juice expressed from thawed pea leaves is compared with a preparation from the same leaves after fractionation with ammonium sulphate.

The lower saturation rate with the crude material suggested the presence of an inhibitor but no evidence of this could be obtained by addition of the purified material to the crude juice. The saturation level was then that of the purified protein. It was also noticed that some samples of the pure, or partially purified, protein lost activity on storage, particularly in solution. The loss consisted of a progressive flattening of the rate curve until the maximum $Q_{O_2}^{chl.}$ fell to about half the original value. A change of this kind may imply a decrease in the catalytic activity of the protein molecule rather than the complete inactivation of a proportion of the molecules in the solution. No explanation can yet be offered for these fluctuations in maximum

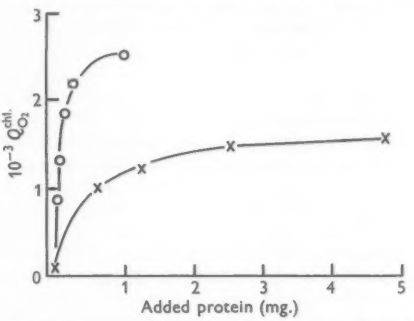

Fig. 2. Rate curves for reduction of metmyoglobin by illuminated pea chloroplasts catalysed by: ×, expressed sap from thawed pea leaves; ○, pea-leaf protein after ammonium sulphate fractionation. Chlorophyll (0·04 mg.) 0·39 μmole of metmyoglobin; 0·066 M-phosphate buffer, pH 7·4, to 3 ml.

$Q_{O_2}^{chl.}$ but their occurrence made invalid a quantitative assay of recovery of protein, in terms of its activity, at the different steps in the preparation. Nevertheless the activity at saturation of a fresh preparation is of the same order as the most efficient hydrogen acceptors in the Hill reaction yet described. Maximum $Q_{O_2}^{chl.}$ values of 2700 were commonly observed under the assay conditions described.

The efficiency of the plant protein in catalysis is illustrated in Fig. 3. Here the rate curve of reactions with an electrophoretically purified preparation is expressed in terms of m-μmoles of the leaf protein calculated in terms of the mol.wt. of 19 000. When the chloroplast system was 75% saturated, 0·39 μmole of metmyoglobin underwent reduction in the presence of 3 μm-moles of leaf protein at a rate corresponding to $Q_{O_2}^{chl.}$ 1550.

Absorption spectrum. After paper electrophoresis the active fraction *A* from all the plants examined was pinkish orange in colour. The absorption spectra of preparations from different species resembled each other closely (Fig. 4). In every case absorption in the visible range increased towards the shorter wavelengths with two diffuse absorption bands at 465 and 422 mμ. Denaturation by heating the solution to 60° for 5 min. was accompanied by the disappearance of the two absorption bands to give a clear-yellow solution devoid of metmyoglobin-reducing activity. In a similar way loss of activity occurring spontaneously in stored preparation was always attended by this colour change. It would therefore appear likely that the chromophore, whatever its nature, is not a mere contaminant of the protein.

Origin of the oxidation–reduction properties. The high activity of the protein in catalysing hydrogen transport led us to seek evidence for the presence of substances commonly associated with such activity. The method of Crammer (1948) was followed in an attempt to demonstrate flavin. On adjusting a solution containing 5·4 mg. of the electrophoretically purified protein to pH 2·0 the colour was completely discharged. The acidified solution was allowed to stand for 5 min. and the denatured protein removed by centrifuging after addition of solid ammonium sulphate to 70% saturation. The supernatant was extracted with phenol, and, after the addition of water to the separated phenol layer, ether was added to return any flavin present to the aqueous phase. Examination of this phase (1·2 ml. total volume) in ultraviolet light revealed no fluorescence. Crammer (1948) reported that 0·01 μg. of flavin can be detected by the characteristic green fluorescence. The method is therefore adequately sensitive to detect flavin present in the original material if it occurred as 1 molecule of flavin/molecule of protein (mol.wt. 19 000).

Kearney & Singer (1955), however, were unable to remove flavin from their preparations of succinic dehydrogenase by boiling or cold acid treatment. They had recourse to a preliminary digestion of their material with trypsin and chymotrypsin. This method was followed with the leaf protein but again no fluorescence could be detected in the final aqueous extract. From these observations we con-

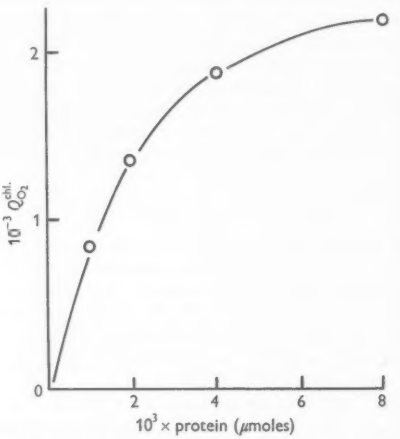

Fig. 3. Rate curve for reduction of metmyoglobin by illuminated pea chloroplasts with an electrophoretically purified preparation of pea-leaf protein as catalyst. Added protein is expressed as μmoles, assuming mol.wt. 19 000; 0·04 mg. of chlorophyll; 0·39 μmole of metmyoglobin; 0·066 M-phosphate, pH 7·4, to 3 ml. Temp., 15°.

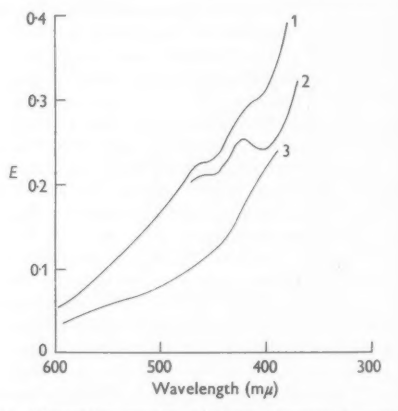

Fig. 4. Absorption spectra of electrophoretically purified leaf protein (fraction *A*). 1, Protein from *Lamium album*; 2, protein from *Pisum sativum*; 3, protein from *L. album* after denaturation at 60° for 5 min.

cluded that either flavin is absent from the protein or it occurs in a tightly bound form.

Attempts were also made to demonstrate the presence of haematin. Impure preparations may contain cytochrome b_3, but after electrophoresis no haem could be detected, as pyridine haemochromogen, in any of the active preparations examined. In a more critical observation 7 mg. of the freeze-dried purified protein in a 1 cm. optical cell was dissolved in 0·5 ml. of 30% pyridine in 0·1N-sodium hydroxide. After the addition of a little sodium dithionite the solution was examined with a small-dispersion spectroscope but no haemochromogen bands could be detected. The method will readily detect 0·25 μmole of haem, and since 7 mg. of the protein corresponds to 0·37 μmole it was concluded that haem could not account for the oxidation–reduction properties of the protein.

As we have reported above, the colour of an active preparation is altered by denaturation and is discharged at pH 2·0. If the acid solution is adjusted to pH 10·0 colour reappears and the solution is then bright yellow. This reaction points to a flavonoid pigment as a source of the colour in the active material but further work is necessary to establish its nature.

Inhibitors of metmyoglobin reduction

It was shown in the earlier work (Davenport *et al.* 1952) that inhibitors of the type of 1:10-phenanthroline and urethane, effective in the Hill reaction with artificial hydrogen acceptors, also inhibit the chloroplast–leaf protein–metmyoglobin reaction. In an attempt to find an inhibitor specific to the leaf-protein part of this reaction we examined the effect of *p*-chloromercuribenzoate and phenylmercuric acetate.

The effect of phenylmercuric acetate on metmyoglobin reduction with a partially purified leaf protein is shown in Fig. 5. Incubation of the protein for 5 min. with 75 μM-phenylmercuric acetate resulted in a reduction in the reaction rate to 20%. Activity was restored to 70% of the uninhibited rate by a further incubation period of 5 min. in the presence of 750 μM-cysteine. Similar results were obtained with *p*-chloromercuribenzoate. Although these results suggested that the action of the mercurial compounds is on thiol grouping in the protein, the integrity of these being essential for activity, this interpretation must be accepted with caution since more prolonged incubation of the protein with either phenylmercuric acetate or *p*-chloromercuribenzoate was found to lead to irreversible inactivation.

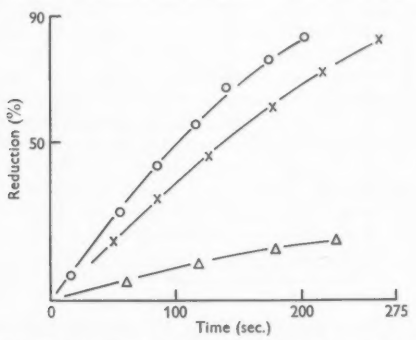

Fig. 5. Reduction of metmyoglobin by illuminated pea chloroplasts in the presence of partially purified pea-leaf protein, showing inhibitory effect of phenylmercuric acetate. ○, No inhibitor; △, 75 μM-phenylmercuric acetate for 5 min. before illumination; ×, 75 μM-phenylmercuric acetate for 5 min. followed by 740 μM-cysteine for a further 5 min. before illumination.

Although we knew of no reports that organic mercurial compounds are effective in inhibiting the Hill reaction it was necessary to confirm this and also to show that the inhibitory effect was not due to a reaction with the metmyoglobin component of our system. Two series of control experiments were therefore carried out. In the first it was found that neither phenylmercuric acetate nor *p*-chloromercuribenzoate affected the rate at which 2:6-dichlorophenolindophenol was reduced by chloroplasts in the light. In the second series ferric oxalate replaced the leaf protein as catalyst of the reduction of metmyoglobin by the illuminated chloroplasts (Hill & Scarisbrick, 1940). Here again neither phenylmercuric acetate nor *p*-chloromercuribenzoate affected the reaction velocity. The effect of the mercurial compounds appeared therefore to be specific for the leaf-protein part of our reaction system.

Specificity of the hydrogen-transferring activity

In this and the earlier work metmyoglobin has been used as a convenient indicator of the activity of leaf-protein fractions without reference to other possible hydrogen acceptors, either natural or artificial. We have now examined the reactivity of the 'methaemoglobin-reducing factor' towards a number of other hydrogen acceptors including reagents widely used as oxidants in the Hill reaction.

Cytochrome c. Holt (1950) found that cytochrome *c* was reduced by chloroplasts in the light and that reoxidation, mediated by cytochrome oxidase in the chloroplast preparation, occurred in the dark. We have confirmed this observation and have found also that fractions from leaves, active in catalysing the reduction of metmyoglobin, greatly stimulate the rate of reduction of cytochrome *c*. Fig. 6 illustrates a typical experiment with pea

chloroplasts. In the absence of added leaf protein reduction proceeded slowly and was half complete in 200 sec. Further illumination carried the degree of reduction to 75% in 500 sec. In the presence of a crude preparation of the leaf protein reduction was half complete in 50 sec. and continued apparently to completion. Fig. 7 indicates the presence of an active cytochrome oxidase in the pea chloroplasts used. With added leaf protein reduction proceeded almost to completion but in darkness reoxidation occurred. With the comparatively stable chloroplasts from peas this cycle of alternate reduction and oxidation could be repeated many times. The dark oxidation was shown to be inhibited by cyanide. Reduction in light was stimulated by this reagent, showing that the oxidase also operated in the light. In the absence of cyanide the rate of reduction of cytochrome *c* by illuminated pea chloroplasts thus represents a net balance between the forward and backward reactions.

To determine the efficiency of the purified leaf protein in promoting the reduction of cytochrome *c* it was desirable to use a chloroplast preparation substantially free from cytochrome-oxidase activity. Carefully fractionated chloroplasts from leaves of spinach beet were found to meet this requirement. In a typical experiment, illustrated in Fig. 8, reduction without added leaf protein is slow ($Q_{O_2}^{chl.}$ 150). The addition of 2 μm-moles of a purified pea-leaf protein (fraction *A* after electrophoresis) greatly stimulated the reaction rate to the very high $Q_{O_2}^{chl.}$ value of 3800. This, so far as we are aware, is the highest reaction rate yet reported for a chloroplast reaction. No significant oxidation of the reduced cytochrome took place after 200 sec. darkness, indicating the relative freedom of this preparation of chloroplasts from cytochrome oxidase.

Cytochrome b_3. The auto-oxidizable cytochrome component b_3 was detected by Hill & Scarisbrick (1951) in both the aerial and underground organs of higher plants and it was extracted by them from leaves and partially purified. In the present work a small quantity of the cytochrome was accumulated as a by-product of the electrophoresis separation of the active leaf protein. Freeze-dried preparations of fraction *C* from the paper were dissolved in a little water. The amount available was too small for the setting up of comparison standards of the oxidized and reduced cytochromes in the cups of the spectrocolorimeter. Solutions of globin haemochromogen and globin parahaematin were therefore

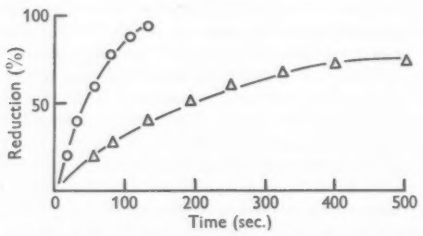

Fig. 6. Reduction of cytochrome *c* by illuminated pea chloroplasts, showing effect of adding partially purified pea-leaf protein. △, No added protein; ○, 0·2 mg. of protein added before illumination. Chlorophyll (0·05 mg.); 0·05 μmole of cytochrome *c*; 0·06 M-phosphate, pH 7·2, to 3 ml. total vol.

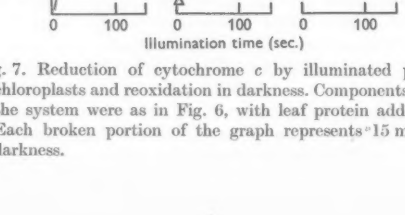

Fig. 7. Reduction of cytochrome *c* by illuminated pea chloroplasts and reoxidation in darkness. Components of the system were as in Fig. 6, with leaf protein added. Each broken portion of the graph represents 15 min. darkness.

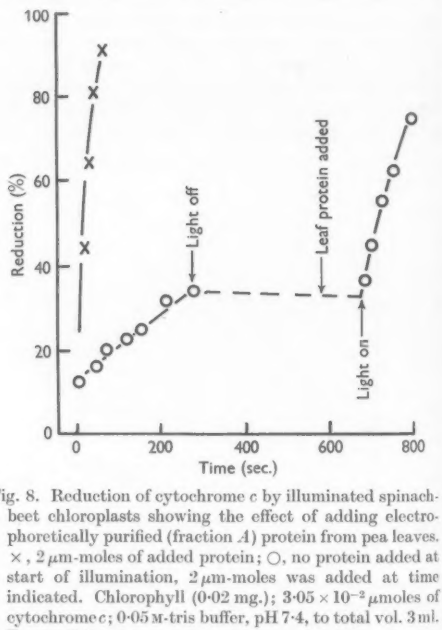

Fig. 8. Reduction of cytochrome *c* by illuminated spinach-beet chloroplasts showing the effect of adding electrophoretically purified (fraction *A*) protein from pea leaves. ×, 2 μm-moles of added protein; ○, no protein added at start of illumination, 2 μm-moles was added at time indicated. Chlorophyll (0·02 mg.); $3{\cdot}05 \times 10^{-2}$ μmoles of cytochrome *c*; 0·05 M-tris buffer, pH 7·4, to total vol. 3 ml. Temp., 17°.

used instead. It was found that the spectrum of the haemochromogen was sufficiently similar to that of the reduced cytochrome for valid measurements to be made.

When cytochrome b_3 was illuminated in the presence of washed pea chloroplasts no bands of the reduced cytochrome could be detected in 1 min. The addition of 0·1 ml. of an active fraction of the leaf protein resulted in the immediate appearance of the α and β bands of the reduced cytochrome at 559 mμ and 530 mμ respectively. The reaction proceeded in 20 sec. to 70% completion. In the dark, reoxidation occurred in less than 10 sec. and the cycle of reduction and reoxidation was repeated ten times in a 30 min. period. It was concluded that the 'methaemoglobin-reducing factor' is highly active in catalysing the reduction of cytochrome b_3 by illuminated chloroplasts.

Heart-muscle preparation. The efficiency of the 'methaemoglobin-reducing factor' in hydrogen transport between the insoluble chloroplast and soluble haem–protein compounds led us to investigate the possibility that similar hydrogen transport could occur between two insoluble systems. A suitable particulate preparation to act as hydrogen acceptor proved to be the standardized heart-muscle preparation of Keilin & Hartree (1947). A preparation of this type (kindly presented to us by Dr E. F. Hartree) represents physically disrupted mitochondria still retaining an active succinoxidase system.

The spectroscopic observations were carried out in tubes of effective light path 1·8 cm. and containing 2 ml. of the heart-muscle preparation and washed pea chloroplasts (0·05 mg. of chlorophyll), made up to 6 ml. with 0·05 M-phosphate, pH 7·5. No reduction of cytochrome could be detected either in the presence or the absence of added leaf protein. This result was expected since the highly active cytochrome oxidase in the preparation would mask a low rate of cytochrome reduction. The cytochrome oxidase was therefore partially inhibited by cyanide at a concentration of 0·16 mM. In the presence of cyanide after 20 sec. illumination of a tube with added leaf protein, the bands of cytochrome *a*, *b* and *c* could be readily observed; cytochrome *b* was reduced to a much smaller extent than cytochrome *c*. In the absence of the active leaf protein no reduction occurred. In darkness reduced cytochrome was reoxidized in less than 10 sec. The 'methaemoglobin-reducing factor' will thus catalyse hydrogen transport between the insoluble chloroplasts and a particulate preparation containing the respiratory cytochrome system.

Other hydrogen acceptors. The available information about the specificity of the 'methaemoglobin-reducing factor' towards a variety of hydrogen acceptors is shown in Table 1. All the active acceptors are haem–protein compounds.

Specificity towards the hydrogen donor. In all the observations reported above the reducing power of illuminated chloroplasts supplied the necessary potential for active hydrogen transport catalysed by the 'methaemoglobin-reducing factor'. The reactivity of the protein suggests an analogy with the coenzyme-linked cytochrome *c* reductases. We therefore attempted to replace the illuminated chloroplast by reduced coenzymes I and II.

Coenzyme I was reduced by a lactic dehydrogenase preparation from heart muscle (kindly provided by Dr V. Massey). The activity of the enzyme was confirmed by following the appearance of the band at 340 mμ in the Beckman DU spectrophotometer with a test system containing 3 μmoles of coenzyme I, 75 μmoles of sodium lactate, 0·02 ml. of the enzyme and 0·06 M-phosphate, pH 8·0, to 3 ml. The enzyme was added to start the reaction.

In a similar system, where 0·4 μmole of metmyoglobin and 10 μm-moles of active leaf protein replaced part of the phosphate buffer, no reduction of the metmyoglobin occurred. A similar negative result was also obtained with cytochrome *c*. At the end of the observation washed pea chloroplasts were added and the reaction cell was illuminated. Rapid reduction was observed in both haem–proteins.

A parallel experiment was set up with reduced coenzyme II. Here the coenzyme was reduced by purified *iso*citric dehydrogenase from baker's yeast (the gift of Miss J. Moyle). The system contained 0·2 ml. of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (0·5 M; pH 7·4), 0·1 ml. of $MnCl_2$ (2 mM), 0·2 ml. of coenzyme II (0·125 mM), 0·1 ml. of *iso*citrate (0·01 M) and water to 3 ml. Increments in density at 340 mμ were observed. Here also the addition of metmyoglobin or cytochrome *c* to the system, either in the presence or the absence of active leaf protein, did not lead to reduction of the haem–protein. Chloroplasts subsequently added

Table 1. *Specificity of the 'methaemoglobin-reducing factor' towards various hydrogen acceptors in the Hill reaction*

Rate of reduction by an illuminated chloroplast system stimulated by added leaf protein	Rate of reduction by an illuminated chloroplast system not stimulated by added leaf protein
Methaemoglobin	Ferricyanide
Metmyoglobin	Ferric oxalate + ferricyanide
Cytochrome *c* (in solution)	2:6-Dichlorophenolindophenol
Cytochrome b_3 (in solution)	Methylene blue
Washed heart-muscle prep. (in the presence of CN^-):	
Cytochrome *a*	
Cytochrome *b*	
Cytochrome *c*	

led to a rapid reduction on illumination. The 'methaemoglobin-reducing factor' is thus not able to accept hydrogen from reduced coenzyme in the manner of a coenzyme-linked reductase.

Location of the 'methaemoglobin-reducing factor' in the cell

The ease with which 'methaemoglobin-reducing' activity passes into solution when leaves are disrupted would suggest that the activity is not associated with a particle in the cell. However, cytochrome *c* (Hill & Scarisbrick, 1951), presumably arising from leaf mitochondria, and cytochrome b_3, stated by Martin & Morton (1957) to be microsomal in origin, may appear in solution under conditions suitable for the extraction of the active leaf protein. A particulate origin for the active protein cannot therefore be excluded. The observation that activity is confined to green tissues (Davenport *et al.* 1952) and the great reactivity of the protein towards chloroplasts led us to look at the possibility that extracts of isolated chloroplasts might possess some activity.

Leaves of *Lamium album* (130 g.) were ground in 500 ml. of 0·5 M-glucose containing 0·03 M-phosphate, pH 7·4. The chloroplasts were obtained by centrifuging and were washed once in the glucose medium and converted into an acetone-dried powder. The powder was extracted with water, filtered and the filtrate diluted to a volume such that 1 ml. was equivalent to 1 g. of leaf, based on the original chlorophyll content. A similar extract was prepared from an acetone-dried powder of *Lamium* leaves and adjusted to the same equivalent concentration. With pea chloroplasts and metmyoglobin, it was found that the reaction rate at saturation with respect to added extract was the same with both preparations ($Q_{O_2}^{chl.}$ 1540), but the volume required to reach saturation was three times as great with the chloroplast extract as with the leaf extract. It was clear from this result that chloroplasts contain a 'methaemoglobin-reducing' activity equal to 30 % of that of the whole leaf. It is not yet possible to decide whether in the intact leaf the chloroplasts contain the whole of the active material.

DISCUSSION

The observations reported in this paper show that the activity of leaf extracts in promoting the photochemical reduction of a variety of haem–protein compounds by illuminated chloroplasts can be accounted for in terms of a protein of small molecular weight. Purification of the active material was greatly facilitated by the finding that the bulk of soluble leaf protein in all the species examined was precipitated at 50 % saturation with ammonium sulphate. The albumin fraction, precipitated at over 50 % saturation, was invariably small and since this fraction contained all the haem–protein-reducing activity a considerable measure of purification could be achieved by ammonium sulphate fractionation alone.

The high activity of the purified protein in catalysis is most clearly seen when metmyoglobin is used as hydrogen acceptor. This haem–protein was used here as a convenient reagent, which is not reduced by chloroplasts in the absence of added catalyst, but without reference to the nature of possible hydrogen acceptors *in vivo*. On the other hand, it has been known for some time (Hill & Scarisbrick, 1951) that cytochrome *c* is a constituent of green leaves and that it will serve as hydrogen acceptor in the Hill reaction (Holt, 1950). More recently Jagendorf (1956) has measured the rate of reduction of the cytochrome by chloroplasts and has obtained rates corresponding to $Q_{O_2}^{chl.}$ of 314 and 1370. Under our conditions it was shown that addition of catalytic amounts of the purified leaf protein could bring about a stimulation of the reduction rate by as much as 25-fold.

In the catalysed reduction of metmyoglobin and the other haem–proteins by the illuminated particulate system containing chlorophyll it would be natural to assume that the factor undergoes an oxidation–reduction cycle. On this assumption the factor is acting as a hydrogen acceptor for the chloroplast preparation. From the data presented here it can be concluded that both the rate of reduction and the affinity of the factor for the hydrogen-donating 'end' of the chloroplast particulate material are quite large. This would appear to place the factor in a class apart from any other soluble substance so far found in leaf-tissue extracts. In short it would be concluded that the factor could act in an important path for hydrogen transfer in photosynthesis.

When we consider the substances shown to accept hydrogen (or to be reduced) in the presence of the factor, the relation this process might have to photosynthesis is far from clear. The reduction of cytochrome components would be in general even less favourable from the energetic point of view than the reduction of metmyoglobin. Further, the factor will not act as a cytochrome reductase, nor will it catalyse the reduction of dyes in the so-called Hill reaction. The important property is the specific reaction with the chloroplast material.

In the early experiments (Hill, 1939; Davenport, 1949) with extracts of acetone-extracted leaf powder, oxygen production could be observed to take place rapidly in the presence of chloroplasts in the light. The factor itself would have been present in too small a quantity to give a measurable amount of oxygen under these conditions. These earlier experiments, together with the present work, lead

to the conclusion that the factor can catalyse the reduction of soluble material in the leaf extracts. Further work is necessary to trace the paths of hydrogen transport after the reduction of the factor by the illuminated chloroplast and to determine the nature of the group concerned in the oxidation–reduction cycle.

SUMMARY

1. A protein factor from leaves, previously shown to be active in catalysing the reduction of methaemoglobin and metmyoglobin by illuminated chloroplasts, has been purified by fractionation with ammonium sulphate followed by electrophoretic separation of the active material on paper.

2. The purified protein was found to give a single symmetrical boundary in the ultracentrifuge and in the Tiselius electrophoresis apparatus.

3. The molecular weight of the protein, calculated from sedimentation and diffusion measurements, is 19 000.

4. The purified protein is highly active in catalysing the photochemical reduction of metmyoglobin and also stimulates the reduction of cytochrome *c*, cytochrome b_3 and the cytochrome components present in a particulate heart-muscle preparation.

5. The photochemical reduction is inhibited by organic mercury compounds and this inhibition is partially reversed by cysteine.

6. The protein does not catalyse the reduction of haem–proteins when coenzymes served as hydrogen donor. Illuminated chloroplasts were the only effective hydrogen-donating system observed.

7. The chemical nature of the group conferring oxidation–reduction properties to the protein has not yet been determined but neither flavin nor haem could be detected.

8. Metmyoglobin-reducing activity has been observed in extracts of chloroplasts and was shown to represent one-third of the activity extractable from the whole leaf.

In addition to the acknowledgements made in the text the authors wish to acknowledge the help of Mr F. W. Arnold and the late Mr H. Pentelow in the collection of leaf material, and to thank Mr B. E. Boon for his assistance with the ultracentrifuge runs and Mr B. R. Slater for the diffusion measurements. This work formed part of a programme supported by the Agricultural Research Council.

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The Isolation and Properties of a Proteolytic Enzyme, Cathepsin D, from Bovine Spleen

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Interest in the fate of protein antigens in an antibody-forming organ has led us to investigate the proteolytic enzymes of the spleen. The intracellular proteases (cathepsins) have been studied in many animal tissues, though none has been fully characterized (see Smith, 1951). Those of the spleen were studied by Anson (1939), using his haemoglobin assay (Anson, 1938) to follow the activity, and an extensive series of papers by Bergmann, Fruton and co-workers (summarized by Fruton, 1957–58) describe many properties of cathepsins A, B and C which have been obtained from both spleen and

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kidney. These three enzymes are distinguished from one another by their different specificities by the use of synthetic peptides as substrates. Our original intention was to attempt a survey of the proteolytic activity of spleen to estimate the number of different cathepsins and the relative amounts present. Hence the non-specific haemoglobin assay was used to follow activity. In fact, one enzyme appears to be responsible for about two-thirds of the proteolytic activity (as judged by haemoglobin assay) of a crude spleen extract. This enzyme has therefore been isolated and as it will not hydrolyse any of the typical substrates of cathepsins A, B and C it has been named cathepsin D. As isolated, it occurs in at least ten forms separable from one another by chromatography and electrophoresis, and present evidence suggests that it is present in the spleen in this complexity. The isolation and properties of cathepsin D will be described in this paper.

A preliminary report of this work has been given (Press & Porter, 1958).

EXPERIMENTAL

Chromatography on cellulose ion-exchangers

Reagents. Sodium borate–phosphate, pH 8·4, 5·25 mM: 2·25 mM-$Na_2B_4O_7$ and 3 mM-NaH_2PO_4; 21 mM: 9 mM-$Na_2B_4O_7$ and 12 mM-NaH_2PO_4; 8·75 mM: 3·75 mM-$Na_2B_4O_7$ and 5 mM-NaH_2PO_4; 13 mM: 5·5 mM-$Na_2B_4O_7$ and 7·5 mM-NaH_2PO_4. Sodium phosphate, pH 3·7, 0·5 M with respect to phosphate; sodium phosphate, pH 6·4, 0·01 M with respect to phosphate; sodium acetate, pH 5·5, 0·01 M, 0·05 M and 0·2 M with respect to acetate. Cellulose was Solka-Flok, B. W. 200 mesh; 2-chlorotriethylamine hydrochloride was obtained from Eastman Kodak Ltd.; monochloroacetic acid was reagent-grade; sodium monochloromethane sulphonate was synthesized as described by Porath (1957).

Diethylaminoethylcellulose. Diethylaminoethylcellulose (DEAE) was prepared as described by Peterson & Sober (1956). Solka-Flok cellulose was graded to remove some of the finer particles by sedimenting in water and discarding the material which did not settle in an hour; this was done three or four times until all the remaining cellulose settled in this time. It was then dried by washing on a Büchner funnel with acetone and finally in an oven at 100°. The 2-chlorotriethylamine hydrochloride was recrystallized by dissolving it in the minimum volume of hot methanol, filtering the hot solution and then allowing it to crystallize at 2°. The yield was greatly increased by the addition of about 10–20% of ethyl acetate at this stage, and the crystals were washed with ethyl acetate and dried. DEAE-chromatography columns were prepared as described by Sober, Gutter, Wyckoff & Peterson (1956) by suspending in 5·25 mM-borate–phosphate buffer, adjusting the pH to 8·4 by addition of a few drops of 5 M-NaH_2PO_4, followed by washing in a sintered-glass filter funnel with about 2 l. of buffer when the pH of the eluate and the buffer were exactly the same. The column was then poured and allowed to settle under 2 ft. of water pressure, the column was washed through with buffer overnight and the pH of the eluate again checked. A piece of filter paper was put on top of the column and then the column allowed just to drain and the protein solution applied to the centre of the paper. When the solution had sunk into the column, it was washed in with a few millilitres of buffer and eluted with buffer under a pressure of about 2 ft. of water. Higher pressures were not used as it was found that the column then packed so tightly that the flow rate was reduced. Concentration-gradient elution was carried out by running a buffer of higher concentration from a reservoir into a mixing vessel of constant volume containing the buffer of lower concentration.

After use, DEAE was recovered by washing on a filter funnel with N-NaOH to remove adsorbed protein and then washing with water to neutral pH.

Carboxymethylcellulose. Carboxymethylcellulose (CM) was also prepared according to the method of Peterson & Sober (1956) with graded Solka-Flok and chloroacetic acid. The CM columns were prepared in the same way as for the DEAE columns by first adjusting the pH with strong buffer and then washing with the buffer to which they were to be equilibrated. After use the CM was recovered by washing with a solution containing 0·25 M-NaCl and 0·25 N-NaOH and then with water to neutrality.

The CM and the DEAE could be dried by washing with ethanol and evaporating in a vacuum desiccator over solid NaOH and conc. H_2SO_4, but this is a slow process and both exchangers can be stored quite satisfactorily in water at 2°.

The flow rate of both DEAE and CM columns was about 50 ml./hr. for columns of cross-section 4·3 cm.^{2} and a pressure of about 2 ft. of water. If the flow rate was less than about 25 ml./hr. the cellulose ion-exchanger was graded by sedimenting in water for an hour and discarding the material which failed to settle. After repeating this two or three times, the flow rate became satisfactory.

Sulphomethylcellulose. Sulphomethylcellulose (SM) was prepared from graded Solka-Flok and sodium monochloromethane sulphonate as described by Porath (1957). The preparation contained a large amount of very fine particles, which were removed by decantation. The exchanger was recovered after use by washing it with 0·1 N-NaOH, followed by water to neutrality.

Phosphate and acetate concentration. Phosphate concentration in the eluate from DEAE columns (Fig. 1*a*) was determined by the method of Martland & Robison (1926). Acetate concentration in the eluate from CM columns, Figs. 3, 4, 6 and 7, was calculated from the formula

$$\log (C_1 - C_v) = \frac{-v}{2{\cdot}3 \times V_0} + \log (C_1 - C_0),$$

where C_1 is the concentration in the reservoir, C_v is the concentration in the mixing vessel when v ml. has run in, V_0 is the volume of the mixing vessel, and C_0 is the original concentration in the mixing vessel.

Electrophoresis

Zone electrophoresis. Zone electrophoresis was performed in a cellulose column as described by Porath (1956) with 0·02 M-sodium acetate, pH 5·6, for 24 hr. at 4–5 v/cm.

Starch-gel electrophoresis. Buffers: 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 9·1, used in the starch gel, contained 4·2 g. of tris, 0·42 g. of ethylenediaminetetra-acetate and 0·32 g. of boric acid in 1 l. of

solution (Aronsson & Grönwall, 1958). The tris buffer for the bridge solution was three times as concentrated as that in the gel. Sodium acetate buffer, pH 5·5, was 0·03 M for the starch gel, and 0·3 M for the bridge solution.

Potato starch (British Drug Houses Ltd.) was hydrolysed in acidic acetone, as described by Smithies (1955), for 2¾ hr. at 38·5°; the reaction was stopped by addition of sodium acetate, the starch filtered and washed with 5 l. of water, followed by 1 l. of borate buffer (0·3 M), pH 8·0, followed by washing with a further 2 l. of water and then washing with acetone and drying at 45° overnight. Amido Black (George T. Gurr Ltd., London, S.W. 6) for staining starch gel: 1·6 % (w/v) in a solvent consisting of methanol–water–acetic acid (50:50:10, by vol.). Wash solution for developing stain: methanol–water–acetic acid (50:50:10, by vol.).

Starch-gel electrophoresis was carried out as described by Smithies (1955) and Poulik & Smithies (1958). Hydrolysed potato starch was used at a concentration of 14 % (w/v); the gel was prepared and poured into a Perspex tray 25 cm. × 4 cm. and 6 mm. deep and allowed to set for 1 hr. or overnight. Electrophoresis in the tris buffer was for 16 hr. at 14 mA and 8–2 v/cm. In acetate buffer the current was 14 mA for 15 hr. at 3–2 v/cm. Calomel electrodes were used and contact was made with the gel through a bridge solution by strips of Whatman no. 3 filter paper soaked in the bridge solution. A horizontal slice of the starch was cut and stained with Amido Black for 2 min. and developed by washing for several hours. Up to three different enzymes could be subjected to electrophoresis in the same gel by inserting pieces of Whatman no. 3 filter paper, soaked in 1 % solution of the enzyme, side by side into a vertical slit cut across the gel. It is possible to recover the protein from the unstained slice of the gel by freezing, thawing and centrifuging the sections containing protein. From the solution squeezed out of the gel in this way, recovery of activity was about 30–50 %.

Methods of enzyme assay

Reagents. Armour Laboratories' bovine haemoglobin enzyme-substrate powder was suspended in water and dialysed at 2° for 4–5 days and then diluted with water to 2·5 % (w/v).

Sodium citrate, 0·4 M with respect to citrate, pH 2·8, gives pH 3·0 in reaction mixture with haemoglobin.

Cysteine hydrochloride (0·07 M) was stored at 2°.

Bovine serum albumin was Armour Laboratories' bovine crystallized plasma albumin.

Trichloroacetic acid (A.R.) was 0·3 M and 0·6 M.

N-Acetyl-DL-phenylalanyl-L-di-iodotyrosine, L-tyrosyl-L-cysteine and L-cysteinyl-L-tyrosine were kindly given by Dr R. Pitt-Rivers.

Benzyloxycarbonyl-L-glutamyl-L-tyrosine and glycyl-L-tyrosine amide acetate were supplied by Mann Research Laboratories. Benzoyl-L-arginine amide was synthesized by Mr A. Hemmings.

Assay with haemoglobin. Proteolytic activity was assayed at pH 3·0 by a modification of Anson's (1938) method. Haemoglobin (2·5 %; 4 ml.) was added to 1 ml. of citrate buffer, 1 ml. of cysteine hydrochloride and 1 ml. of enzyme solution and incubated at 37° for 10 min., then 9 ml. of 0·3 M-trichloroacetic acid was added and the mixture was filtered through Whatman no. 3 paper. The absorption of the filtrate at 280 mμ was measured. Reagent blanks were carried out by adding the trichloroacetic acid before the enzyme solutions. One unit of proteolytic activity is that which will give an extinction of the trichloroacetic acid filtrate at 280 mμ of 1·0 in excess of the blank reading. There is a linear relationship between the amount of enzyme used and the absorption of the trichloroacetic acid filtrate, up to an extinction of 0·3, and all assays were done within this range.

Assay with acid-denatured haemoglobin. Haemoglobin (2·5 %) was incubated at 37° at pH 2·3 in 0·16 M-citric acid for 1 hr. and by addition of N-NaOH the pH was adjusted to that required for the assay. Cysteine hydrochloride was added so that the final solution contained 1·5 % of haemoglobin and was 0·01 M with respect to cysteine hydrochloride. The method of assay was the same as for the routine assay described above.

Assay with acid-denatured albumin as substrate. Bovine serum albumin (1·7 %) was incubated at 37° for 1 hr. at pH 1·8 in 0·33 M-citric acid. N-NaOH was then added to adjust the pH to that required for the assay, and also cysteine hydrochloride to a concentration of 0·01 M. The final solution contained 0·85 % of albumin; 2·5 ml. of this solution was incubated with 1 ml. of enzyme solution at 37° for 10 min. and then 5 ml. of 0·6 M-trichloroacetic acid was added. The mixture was allowed to stand for 1 hr. at 37°, filtered through Whatman no. 3 paper, centrifuged to give a clear solution and the absorption was read at 280 mμ. Reagent blanks were obtained by adding the trichloroacetic acid before the enzyme.

Assays with peptides. Cathepsin A was assayed with benzyloxycarbonyl-L-glutamyl-L-tyrosine, by the micro-titration method of Grassmann-Heyde at pH 5·0, as described by Tallan, Jones & Fruton (1952). A direct-reading pH meter (Electronic Instruments Ltd.) was used to determine the end-point of the titration, since with crude extracts an indicator was not satisfactory. Cathepsin B was assayed with benzoyl-L-arginine amide as substrate by the method of Greenbaum & Fruton at pH 5·0 (1957). Cathepsin C was assayed by measuring the transamidation reaction between glycyl-L-tyrosine amide and hydroxylamine at pH 7·2 (de la Haba, Cammarata & Fruton, 1955).

The assay with the pepsin substrate *N*-acetyl-DL-phenylalanyl-L-di-iodotyrosine was carried out by incubation of 2 mM-substrate with cathepsin D at a concentration of 0·01 mg. of enzyme N/ml. for 20 min. at 37° at pH 2·0, 3·0 and 5·0. The course of hydrolysis was followed by the ninhydrin reaction described by Cocking & Yemm (1954).

For the assays with L-tyrosyl-L-cysteine and L-cysteinyl-L-tyrosine, the substrate at a concentration of 5 mM was incubated with enzyme, concentration 0·03 mg. of enzyme N/ml., at pH 3·6 for 80 min. at 37°. The digest and controls were evaporated and chromatographed on paper with butanol–acetic acid–water (4:1:5, by vol.) in order to detect any tyrosine liberated.

Hydrolysis of B chain of oxidized insulin by cathepsin D

The B chain of oxidized insulin was prepared according to the method of Sanger (1949), from crystalline insulin (Burroughs Wellcome and Co.). Pyridine–acetate buffer, pH 6·5, contained 10 vol. of pyridine, 0·4 vol. of acetic acid and 90 vol. of water. For paper chromatography, the following solvents were used: butanol–acetic acid (5 vol. of water, 4 vol. of butanol and 1 vol. of acetic acid were

shaken together and allowed to settle; the top phase was used to develop the chromatogram); phenol–ammonia (80%, w/v, phenol in water containing 0·01 mM-ethylenediaminetetra-acetate was used to develop the chromatogram in an atmosphere containing ammonia vapour. For staining, 2% (w/v) ninhydrin in aq. 90% ethanol containing 1% of pyridine was used.

A sample (5 mg.) of B chain of oxidized insulin was incubated at pH 3·0 and 37° with 1 unit of enzyme for 2 and 20 hr. and the digestion was stopped by evaporation in a vacuum desiccator. The dry hydrolysate was dissolved in a few drops of water and applied as a thin line 6 cm. long to Whatman no. 4 paper for electrophoresis at pH 6·5 in pyridine–acetate buffer, by the technique of Michl as described by Ryle, Sanger, Smith & Kitai (1955). A potential of 25 v/cm. was applied across the paper for 3 hr. Strips of paper were stained with ninhydrin, and the portions containing the basic and acidic peptides were eluted with 6N-HCl and hydrolysed in sealed tubes at 105° for 18 hr. The hydrolysates were vacuum-dried several times and chromatographed on Whatman no. 1 paper, butanol–acetic acid and phenol–ammonia solvents being used to identify the component amino acids of these peptides. The neutral peptides were chromatographed on Whatman no. 4 paper with butanol–acetic acid solvent and the peptides thus separated were hydrolysed with 6N-HCl and chromatographed as for the basic and acidic peptides.

Ultracentrifugal analysis

The buffer was sodium phosphate (I 0·2, pH 6·75). Molecular weights were determined by sedimentation in the ultracentrifuge by the Archibald (1947) procedure as described by Charlwood (1957).

The N-terminal amino acid of the enzyme was determined by the fluorodinitrobenzene method of Sanger (1945), with paper chromatography with 2-methylbutan-2-ol–phthalate solvent to separate the dinitrophenyl (DNP) amino acids, as described by Porter (1957).

The concentration of protein solutions was determined by measuring their absorption at 280 mμ.

RESULTS

Preparation of spleen extracts

Bovine spleens were put into solid CO_2 immediately after removal from the animal and kept frozen until used. They were partially thawed, freed from skin and connective tissue and cut for mincing. A single spleen may yield from 400 to 800 g. of minced tissue. A variety of methods of pretreatment and extraction were tried and these are listed in Table 1. When portions of the same spleen were extracted by the different methods or combinations of these methods the yields of proteolytic activity, as judged by haemoglobin assay, all agreed within experimental error and the yield in the supernatant fluid after centrifuging at 40 000 g was the same as in the whole suspension. It appeared therefore that all the proteolytic activity of the spleen is free in the cells or, more probably, is associated with easily ruptured particles such as lysosomes (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). If any of the proteolytic enzymes exist in the cell as inactive zymogens, the activation occurred too rapidly to be observed. The pH optima of these crude extracts, as of purified cathepsin D, lay in the range pH 3·0–4·0 with protein substrates. No evidence of proteolytic activity at neutral or alkaline pH could be detected with the methods of assay used.

Purification of cathepsin D

For routine preparation the minced spleen was extracted by stirring overnight at 2° with twice the volume of 0·1M-sodium acetate saturated with toluene. The purification procedure for 1 kg. of minced spleen is summarized in Table 2. The yield of proteolytic activity varied considerably from spleen to spleen, 5000–14 000 units of proteolytic activity/kg. being obtained in the course of some 12 preparations.

The crude extract was filtered through four layers of cheese cloth and then centrifuged at 40 000 g in a refrigerated continuous-flow Sharples centrifuge. The supernatant was brought to pH 4·6 by adding to 4 vol. of supernatant 1 vol. of acetate buffer (23·4 ml. of acetic acid and 50 ml. of 2M-sodium acetate made to 1 l. with water). The pH was adjusted to pH 4·6 with acetic acid or sodium hydroxide if necessary. A large precipitate formed, which, after settling for at least 1 hr., was removed by centrifuging (1000 g) in a refrigerated bucket centrifuge at 2°. The precipitate was suspended in an equal volume of water and again centrifuged, the supernatants being combined. Between 70 and 90% of the activity was recovered, a sufficiently high figure to suggest that the loss was probably mechanical rather than due to the separation of a distinct enzyme which was insoluble or unstable under these conditions.

Table 1. *Methods of extraction of ox spleen*

All procedures were carried out at 2° except where otherwise stated.

Pretreatment of spleen

- Homogenization for 10 min.
- Stirring overnight
- Acetone-dried powder
- Repeated freezing and thawing
- Incubation of whole spleen at 20° for 3 hr.

Solvents for extraction

- Water
- 0·15M-NaCl solution
- 0·1M-Citrate buffer, pH 5·9 and pH 7·5
- 0·1M-Phosphate buffer, pH 7·0
- 0·1M-Sodium acetate
- 0·1M-Phosphate buffer, pH 7·5, containing 20% of butanol

Table 2. *Preparation of cathepsin D from 1 kg. of minced spleen*

All procedures were carried out at 2°.

		Volume (ml.)	Total enzyme units/kg. of spleen		Enzyme units/mg. dry wt.
Whole suspension in 0·1 M-sodium acetate		3200	9600		—
Supernatant from centrifuging at 40 000 *g*		2900	8000		0·07
pH 4·6 supernatant		4500	6300		0·2
Precipitate with 90% saturated ammonium sulphate resuspended and dialysed		400	6000		0·3
Pressure-dialysed solution before chromatography on CM at pH 6·4		70	5900		0·3
Eluate recovered from CM column		120	5750		0·5
Pressure-dialysed solution before chromatography on DEAE at pH 8·4		45	5600		0·5
Eluate recovered from DEAE at pH 8·4		600	3800		3·0
Eluate recovered from second run on DEAE at pH 8·4	α	200	950		10
	β	190	940	Total 3760	10
	γ	200	730		7
	δ	250	1140		5
Eluate recovered from chromatography of β form on CM at pH 5·5	β_1	60	90		21
	β_2	50	200		21
	β_3	90	100	Total 700	19
	β_4	60	200		20
	β_5	75	110		16

Solid ammonium sulphate was added to the combined supernatants at pH 4·6 and 2°, to give a 90% saturated solution (615 g./l.). The suspension was filtered through Whatman no. 50 paper with suction and the filter cake was resuspended in as small a volume of water as possible and dialysed overnight against running tap water in the cold room. This was primarily a concentration step but some purification was also achieved without loss of activity. Any precipitate remaining was centrifuged at 1000–2000 *g* at 2°, washed with an equal volume of water and the combined supernatants were dialysed under pressure against 0·01 M-sodium phosphate buffer, pH 6·4, for 3 days at 2°. The concentrated solution, adjusted to pH 6·4 with a few drops of acid or alkali if necessary, was run slowly on to a 20 g. CM column (30 cm. × 4·3 cm.²) equilibrated with 0·01 M-phosphate buffer, pH 6·4. Under these conditions the proteolytic activity is not held on the column and was recovered quantitatively in the eluate. Much heavily pigmented inactive material was adsorbed and discarded. The enzymically active eluate was concentrated by pressure dialysis against sodium borate–phosphate buffer (5·25 mM, pH 8·4). The solution (about 50 ml.) was chromatographed on a DEAE column 30 cm. × 4·3 cm.², equilibrated with the same buffer. The remaining pigment was held at the top of the column; some inactive protein was unadsorbed and emerged at the solvent front, followed by the proteolytic activity. A buffer concentration gradient was used with a 250 ml. mixing vessel, and a reservoir containing 21 mM borate–phosphate, pH 8·4. About 70% of the proteolytic activity was recovered.

Cathepsins B and C, assayed by using their specific substrates, were not eluted under these conditions but could be partially recovered by subsequent step-wise elution with 0·5 M-sodium phosphate buffer, pH 3·7. Only 20% of cathepsin C and 50% of cathepsin B originally present were recovered in this procedure, and it is clear that this would be an unsatisfactory method of preparation for these enzymes. The eluate containing cathepsins B and C had about 10% of the total proteolytic activity, giving an overall loss of 20%, which is probably accounted for by the loss of the B and C enzymes. Cathepsin A assays on the initial spleen extracts showed only minimal amounts to be present in all preparations and none could be detected after the first stage in purification. No explanation has been found for this discrepancy between our results and those of Fruton & Bergmann (1939).

The cathepsin D fraction was concentrated by pressure dialysis and run again on a DEAE column under similar conditions but with a slower salt gradient. With a column 30 cm. × 4·3 cm.², initial buffer 5·25 mM borate–phosphate, pH 8·4, and a reservoir buffer concentration 21 mM, pH 8·4, the volume of the mixing chamber was increased from 250 ml. to 1 l., thus giving a more gentle gradient. The first DEAE column could be omitted if much lower loads were used but in practice it was found preferable to run again on a second DEAE column, as described, and work with high loads. The elution

diagram is shown in Fig. 1*a*, and it can be seen that there are four enzymically active components, labelled α, β, γ and δ in order of their elution from the column. The recovery of activity from this column is near 100%, suggesting that the losses on the first DEAE column are due to losses of cathepsins B and C. The specific activity of the cathepsin D enzymes varies from preparation to preparation in the range 3–10 units of proteolytic activity/mg. If the activity was below 8 units/mg. the enzymes were run again on DEAE by simple elution chromatography, i.e. at constant salt and pH with 8·75 mM-borate–phosphate buffer, pH 8·4, for the enzymes α, β and γ and 13 mM buffer, pH 8·4, for the δ enzyme. Satisfactory chromatography was obtained under these conditions and gave further purification, as illustrated for the α enzyme in Fig. 2.

It was apparent, however, from starch-gel electrophoresis and ultracentrifuge studies that none of the four enzymes was pure, and chromatography on CM was now used to get further purification. The conditions chosen, which were applicable to all the enzymes, were to put up to 400 units of proteolytic activity on a 25 cm. × 1 cm.² column with an initial buffer concentration of 0·01 M-sodium acetate pH 5·5. After 2 column vol. a gradient was applied with a constant-volume mixing vessel of 250 ml. and a reservoir buffer concentration of 0·05 M-acetate, pH 5·5. After a further 12 column vol. the concentration of the reservoir buffer was changed to 0·2 M-acetate, pH 5·5. The resulting chromatogram with α enzyme is shown in Fig. 3.

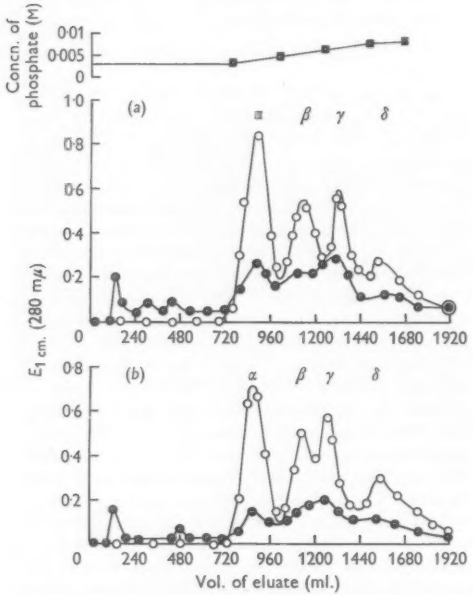

Fig. 1. (*a*) Chromatography of cathepsin D (2000 units of proteolytic activity) in a DEAE column 30 × 4·3 cm.² by gradient elution with sodium borate–phosphate buffer, 5·25–21 mM, pH 8·4. Volume of mixing chamber, 1 l. (*b*) Chromatography on DEAE of a part of the same preparation as in (*a*) (1700 units) which had been left at pH 4·6 and 2° for 1 week before precipitation with ammonium sulphate. ●, Extinction coefficient; ○, units/0·2 ml.; ■, phosphate concentration in eluate.

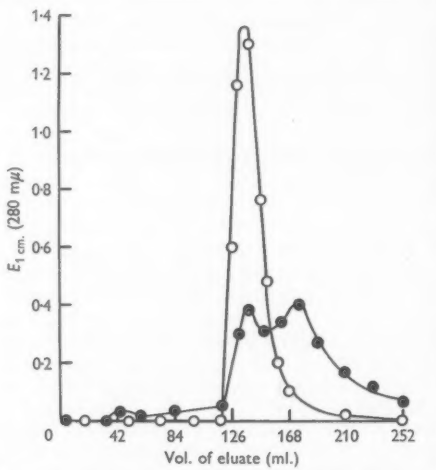

Fig. 2. Rechromatography of α enzyme (150 units) on a DEAE column 30 × 1·5 cm.² at constant buffer concentration and pH (sodium borate–phosphate buffer, 8·75 mM, pH 8·4). ●, Extinction coefficient; ○, units/0·2 ml.

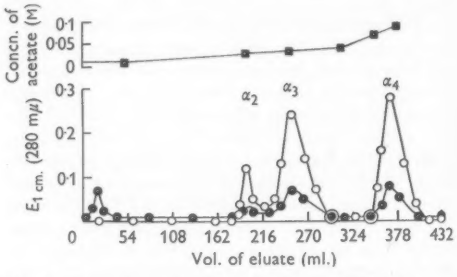

Fig. 3. Chromatography of the α enzyme (150 units) on a CM column 25 × 1 cm.² by gradient elution with sodium acetate buffer, 0·01–0·2 M, pH 5·5. Volume of mixing chamber, 250 ml. ●, Extinction coefficient; ○, units/0·2 ml.; ■, acetate concentration in eluate.

Three active peaks α_2, α_3 and α_4 appear. With β enzyme (Fig. 4) five active peaks are found; with the γ, four peaks, and with the δ, again five. The recovery of proteolytic activity is about 70%. Evidence presented below suggested that the majority of these different forms of cathepsin D were pure.

Evidence for the existence of cathepsin D in at least ten different forms

The validity of the apparent existence of many forms of this enzyme was tested both by further chromatographic studies and by starch-gel electrophoresis (Smithies, 1955). The α, β and γ groups of enzymes ran and ran again at characteristic rates on DEAE columns under constant conditions of salt and pH, as illustrated in Fig. 5. The δ enzyme trails very badly at this concentration but, with 13 mM-borate–phosphate, chromatography on DEAE resolves it into three or four active components. We have not studied these in detail, and so they will be referred to as the δ group. In simple elution chromatography such as this, the same column can be used throughout and technical artifacts are much less likely to arise than in gradient or stepwise elution (see Porter, 1960).

Conditions for simple elution with CM could not be found and, with gradient elution, repeating the running of individual peaks showed the situation to be complex. Some of these second runs are shown in Figs. 6 and 7. α_2 ran again as α_2 and α_4, α_3 ran again as α_3 and α_4, and α_4 gave α_2, α_3 and α_4. This is interpreted as meaning that α_2 is occurring in two positions, some at α_2 and some at α_4, and that α_3 is behaving similarly. The α_4 will be complex, being a mixture of α_2 and α_3 in their slower-running positions. The β enzymes behave somewhat similarly. β_1 runs again correctly but β_2 gives rise to β_2 and β_4, and β_3 to β_3 and β_5. β_4 in turn gives rise to β_2 and β_4 and β_5 to β_3 and β_5. Similar behaviour is found in the γ and δ group enzymes. For some reason the two and three groups of enzymes each give rise to two zones on the column. The slower of these zones run together as α_4 in the α group and separately as β_4 and β_5 in the β group and also in the γ group as γ_4 and γ_5.

Thus there appear to be two genuinely distinct α enzymes, α_2 and α_3, three β enzymes, β_1, β_2 and β_3, two γ enzymes, γ_2 and γ_3, and three or more of the δ group of enzymes, though the last-named have not been examined in as great detail. A

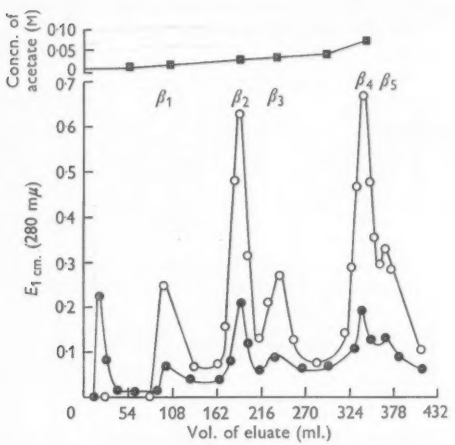

Fig. 4. Chromatography of the β enzyme (370 units) on a CM column 25 × 1 cm.² by gradient elution with sodium acetate buffer, 0·01–0·2 M, pH 5·5. Volume of mixing chamber, 250 ml. ●, Extinction coefficient; ○, units/0·2 ml. ■, acetate concentration in eluate.

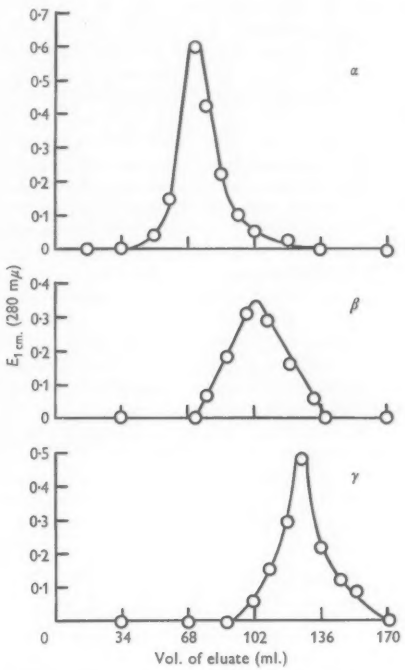

Fig. 5. Rechromatography of α, β and γ enzymes on a DEAE column 23 × 1 cm.² at constant buffer concentration and pH (sodium borate–phosphate buffer, 8·75 mM, pH 8·4). α eluted at R (defined by Martin & Synge, 1941) 0·32, β eluted at R 0·22, γ eluted at R 0·18. ○, Units/0·2 ml.

diagrammatic representation of these results is given in Fig. 8.

The behaviour of these enzymes was now studied in starch-gel electrophoresis and it proved possible to correlate many of the chromatographic results with those obtained with this quite different technique. Thus when purified enzymes are run in starch gel at pH 9·1 (Fig. 9) the most rapidly eluted α enzymes have the lowest mobility, β and γ enzymes move farther but are indistinguishable and

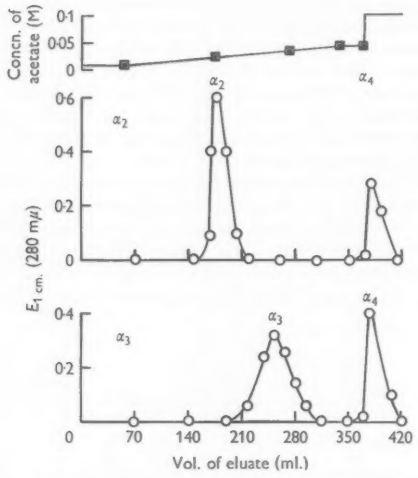

Fig. 6. Rechromatography of α_2 (136 units) and α_3 (155 units) enzymes on a CM column 25 × 1 cm.² by gradient elution with sodium acetate buffer, pH 5·5, 0·01–0·05 M, followed by a step to 0·1 M. Vol. of mixing chamber, 250 ml. ○, Units/0·2 ml.; ■, acetate concentration in eluate.

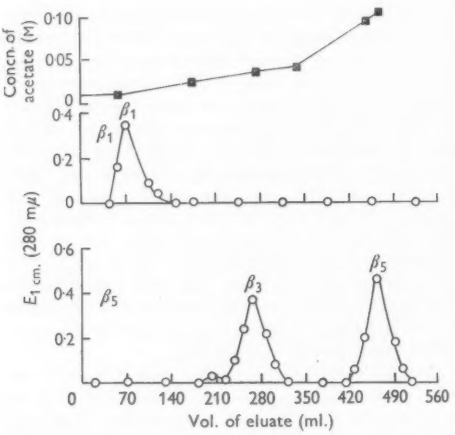

Fig. 7. Rechromatography of β_1 (136 units) and β_5 (250 units) enzymes on a CM column 25 × 1 cm.² by gradient elution with sodium acetate buffer, 0·01–0·2 M, pH 5·5. Vol. of mixing chamber, 250 ml. ○, Units/0·2 ml.; ■, acetate concentration in eluate.

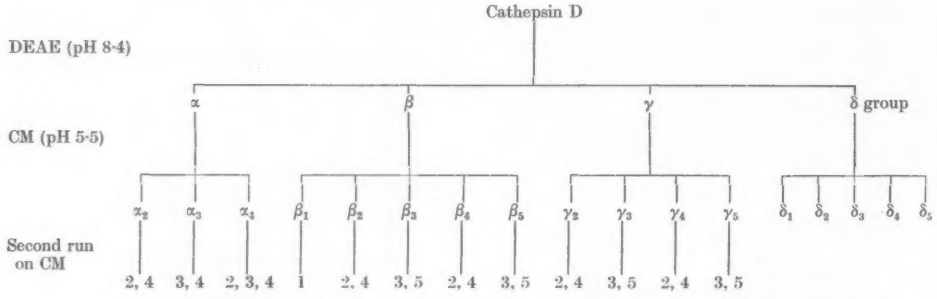
Cathepsin D
DEAE (pH 8·4)
α
β
γ
δ group
CM (pH 5·5)
α_2 α_3 α_4 β_1 β_2 β_3 β_4 β_5 γ_2 γ_3 γ_4 γ_5 δ_1 δ_2 δ_3 δ_4 δ_5
Second run on CM
2, 4 3, 4 2, 3, 4 1 2, 4 3, 5 2, 4 3, 5 2, 4 3, 5 2, 4 3, 5

Fig. 8. Results of chromatography and rechromatography on CM of the α, β, γ and δ enzymes.

the δ group gives three components, the slowest one moving at the same rate as the β and γ. Apparently the δ enzyme is a mixture of several enzymes, as was shown by chromatography on DEAE with 13 mM-borate–phosphate buffer, but the β, γ and slowest δ components are not separable by electrophoresis under the conditions used.

At pH 5·5 (Fig. 10) α_2 and α_3 moved at the relative rates expected from chromatography on CM at pH 5·5, and α_4 proves to be a mixture of α_2 and α_3 as second running on the columns suggested. Similarly with the β enzymes (Fig. 11), 1, 2 and 3 move as expected, 4 runs as 2, and 5 runs as 3. There is therefore no evidence of the double zoning on starch-gel electrophoresis as found in chromatography. If the β enzymes are subjected to electrophoresis at pH 9·1 then they all have the same mobility, in agreement with the chromatographic results. Similarly all the α enzymes have the same mobility at pH 9·1 and are distinct from β enzymes in electrophoresis at the same pH. Thus the starch-gel electrophoresis results agree with the chromatographic results but the double zoning does not occur and the difference between β and γ enzymes at pH 8·4 can be picked out by chromatography but not by electrophoresis.

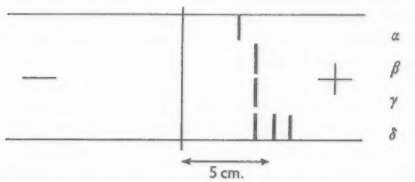

Fig. 9. Starch-gel electrophoresis of cathepsin D enzymes in 35 mM-tris buffer, pH 9·1; 8–2 v/cm. for 16 hr.

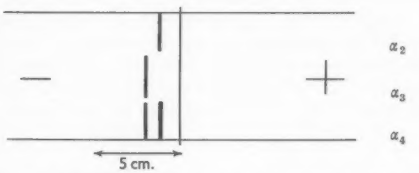

Fig. 10. Starch-gel electrophoresis of α enzymes in 30 mM-sodium acetate buffer, pH 5·5; 3–2 v/cm. for 16 hr.

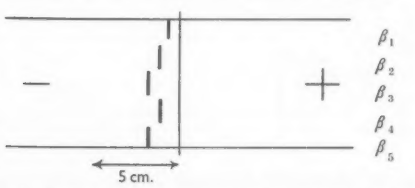

Fig. 11. Starch-gel electrophoresis of β enzymes in 30 mM-sodium acetate buffer, pH 5·5; 3–2 v/cm. for 16 hr.

It therefore appears that cathepsin D can be separated into four or more groups differing in charge at pH 8·4 and that each group can be further subdivided into two or three components differing in their charge at pH 5·5.

Anomalous behaviour of cathepsin D on carboxymethylcellulose at pH 5·5

No satisfactory explanation has been found for the double-zoning phenomena of peaks 2 and 3 of the α, β and γ group of enzymes when run on CM at pH 5·5. At this pH the carboxyl groups of the ion-exchanger are not fully ionized and the percentage ionization is influenced by the salt concentration of the buffer (Peterson & Sober, 1956). Further, this pH is close to the isoelectric point of the protein, which appears to be about pH 6·0. Either of these phenomena might contribute to the anomalous behaviour, but electrophoresis in starch gel as well as chromatography has shown that satisfactory fractionation is dependent on working between pH 5·0 and 6·0. Alternative techniques, such as zone electrophoresis on cellulose columns and chromatography on the ion-exchange resin IRC 50, were tried but without success. Sulphomethylcellulose (Porath, 1957) was prepared, as the more strongly acidic groups are fully ionized at pH 5·5, but similar results to those on CM at the same pH were obtained. At pH 5·0 on SM, satisfactory resolution of the different enzyme peaks was not obtained. Use of other buffers such as citrate or phosphate, instead of acetate, did not affect the chromatographic behaviour, so a careful study was made of the effect of varying the pH between 5·5 and 6·0 with the CM columns. It was found that at pH 5·75 α_2 and β_2 would run as single peaks but that the α_3 and β_3 still gave a second peak in the α_4 and β_5 positions respectively, though less than when running at pH 5·5. At higher pH values some of the enzymes were not retarded on the column. For preparative purposes therefore pH 5·75 appears to be the optimum condition and in the most recent work it has been used for routine preparations.

Purity of cathepsin D fractions

Of the many forms of cathepsin D described only five have been subjected to detailed investigation of their purity and these have been taken principally from the α and β groups. The specific activity of these enzymes was found to be the same, 21 units of proteolytic activity/mg., within the error of the assay.

In starch-gel electrophoresis the enzymes gave only a single band in the two buffers used. As the

high resolving power of this technique is believed to depend on both charge and size (Smithies, 1955), it was expected that these enzymes would appear also to be monodisperse when examined in the ultracentrifuge. This was confirmed for α_3 and β_2. The $S_{20,w}$ was 3·3 and the molecular weight determined by the Archibald procedure was found to be 58 000. The two enzymes were indistinguishable by these criteria.

N-terminal amino acids were estimated by the fluorodinitrobenzene method and both enzymes (α_3 and β_2) gave *N*-terminal glycine, about 0·8 mole/mole of enzyme. There is presumably 1 mole of terminal glycine and the low figure found may arise because of the difficulty of correcting for the loss during hydrolytic liberation of this rather labile dinitrophenyl amino acid. Small amounts (less than 0·1 mole/mole) of terminal glutamic acid were also detectable but whether they arose from a contaminant or as an artifact of the estimation is not known.

Properties of cathepsin D

The properties of several of the α, β, γ and δ enzymes were studied and no differences between them could be found. The results reported are therefore believed to be applicable to all forms of the enzyme.

pH *optima*. The substrates were bovine haemoglobin and bovine serum albumin, both denatured with acid before use, and citrate buffer was used from pH 2·0 to 8·0. The results are given in Fig. 12. With haemoglobin as substrate the optimum is at pH 3·0, and with serum albumin at pH 4·2. As judged by this technique, the hydrolysis of serum albumin is only about one-fortieth of that of haemoglobin. The assay is a measure of the production of peptides soluble in 0·6M-trichloroacetic acid and containing aromatic amino acids. It is unlikely to be directly related to the number of peptide bonds broken. However, the difference in susceptibility of the two substrates is great, so that serum albumin is clearly relatively resistant to hydrolysis by cathepsin D.

Activation of cathepsin D. The haemoglobin activity of the crude spleen extract was increased about 20% by addition of cysteine to 0·01M in the reaction mixture. The assay of pure cathepsin D was not, however, affected by the presence of 0·01M-cysteine, 1 mM-iodoacetamide, 0·1 mM-*p*-chloromercuribenzoate or 1·5 mM-ethylenediaminetetra-acetate in the reaction mixture. It is therefore unlikely that sulphydryl groups play any role in the catalytic activity of the enzyme. The activation by cysteine, observed with the crude enzyme, is lost on the first CM column (Table 2). The disappearance of sulphydryl activation occurs at a step where no proteolytic activity is lost and is presumably therefore not due to the separation of a different sulphydryl-dependent enzyme. It may be that the cysteine is protecting an essential group of cathepsin D against partial inactivation by impurities but that this group is not itself a sulphydryl group.

Cathepsin D was incubated with di*iso*propyl phosphorofluoridate at 100 × mol.prop. of enzyme at pH 3·1 and pH 7·4 for 20 min. at 37° but its activity was unaffected.

Stability. Cathepsin D is heat-labile, all activity being lost after heating at neutral pH at 60° for 40 min. This in contrast to cathepsin C, which is stable under these conditions (Tallan *et al.* 1952). The influence of pH on the stability of the enzyme at 37° was measured and the results are given in Fig. 13. The activity was rapidly lost below pH 2·5 but was unchanged after 2 hr. at pH 6·5. The difference between the stability and pH optimum curves (say at pH 2·5 and 3·5) suggests that instability was due to acid denaturation rather than autolysis, though both may contribute. The loss of activity in acid solutions appears to be irreversible.

Specificity. Six peptides have been tested as substrates for cathepsin D: the substrate for cathepsin A and pepsin, benzyloxycarbonyl-L-glutamyl-L-tyrosine; the substrate for cathepsin B, benzoyl-L-arginine amide; the substrate for cathepsin C,

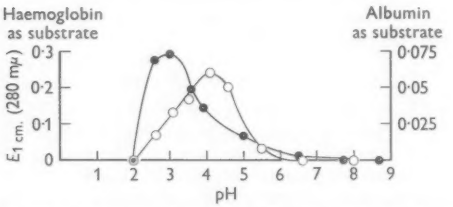

Fig. 12. pH optima of cathepsin D. ●, 0·002 mg./ml. of enzyme; acid-denatured haemoglobin (1·3%) as substrate; ○, 0·02 mg./ml. of enzyme; acid-denatured albumin (0·6%) as substrate.

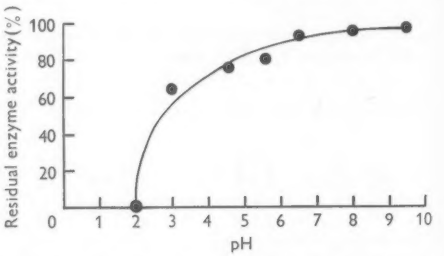

Fig. 13. pH stability of cathepsin D at 37°. ●, Percentage of original enzyme activity recovered after 2 hr. at 37° at different pH values.

glycyl-L-tyrosineamide; the pepsin substrates *N*-acetyl-DL-phenylalanyl-L-di-iodotyrosine, L-tyrosyl-L-cysteine and L-cysteinyl-L-tyrosine. No evidence of hydrolysis could be detected in any of these peptides.

In view of the failure to detect cathepsin A with certainty in the crude spleen extracts, the hydrolysis of benzyloxycarbonyl-L-glutamyl-L-tyrosine was investigated in some detail and the results are summarized in Table 3. At ten times the amount of crude extract used by Fruton & Bergmann some evidence of slight hydrolysis of the substrate was found, but it was still too close to the blank values to be certain that it was significant. Pure cathepsin D did not hydrolyse this substrate. The crystalline enzymes, pepsin and carboxypeptidase, in parallel experiments hydrolysed the peptide and the presence of free tyrosine in the hydrolysis products was confirmed by paper chromatography. It is clear that cathepsin D is unrelated to cathepsin A and the status of the latter enzyme is uncertain.

In view of the failure to find a synthetic peptide substrate for cathepsin D its action on the B chain of oxidized insulin was examined. Incubation was at 37° for 2 and 24 hr. with an enzyme: substrate ratio of 1/100 by wt. After 2 hr. the following peptides were found: 1, $Phe^{1}...Leu^{15}$; 2, $Tyr^{16}...Phe^{24}$; 3, $Tyr^{16}...Phe^{25}$; 4, $Phe^{25}...Ala^{30}$; 5, $Tyr^{26}...Ala^{30}$. [For definition of these abbreviations see the *Biochemical Journal* (1953), **55**, 5.]

After 24 hr. peptides 1, 4 and 5 were still present but peptides 2 and 3 had lost their *N*-terminal tyrosine. In addition the dipeptide Ala^{14}–Leu^{15} and free tyrosine were found. The peptide Phe^{1}–Glu^{13}, present in the 24 hr. hydrolysate, was only just separable from Phe^{1}–Leu^{15} under our conditions.

No difference in the specificities of the different forms of cathepsin D could be found. The results with the B chain are summarized in Fig. 14 and it can be seen that the specificity, though more restricted, is similar to that of pepsin, in spite of the failure of cathepsin D to hydrolyse any of the pepsin substrates tested.

Table 3. *Hydrolysis of benzyloxycarbonyl-L-glutamyl-L-tyrosine at 37°*

Enzyme	Protein N (mg./ml.)	Hydrolysis (%)	Time (hr.)
Crude spleen extract (Fruton & Bergmann, 1939)	0·25	53	2
Crude spleen extract	2·50	10	2
Pure cathepsin D	0·12	None	20
Pepsin	1·4	80	19
Carboxypeptidase	0·07	80	16

Bonds split by pepsin
NH2 NH2
Phe.Val.Asp.Glu.His.Leu.Cys.Gly.Ser.His.Leu.Val Glu.Ala.Leu.Tyr.Leu.Val.Cys.Gly.Glu.Arg.Gly.Phe.Phe.Tyr.Thr.Pro.Lys.Ala
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Bonds split by cathepsin D

Fig. 14. A comparison of the digestion of the B chain of oxidized insulin by pepsin in 0·01 N-HCl (Sanger & Tuppy, 1951) and by cathepsin D in 0·04 M-acetic acid, pH 3·0. Bonds marked with unbroken arrows split rapidly; broken arrows indicate bonds that split more slowly.

DISCUSSION

The purification procedure described for the isolation of cathepsin D is tedious, in spite of the use of chromatographic procedures with a high resolving power and is in contrast with the one- or two-step methods which are successful for other proteins such as insulin (Porter, 1953) and chymotrypsinogen (Hirs, 1953) by similar techniques. It is noteworthy that several proteins will move as one component on DEAE even when simple elution at constant pH and salt concentrations is used (see, for example, the chromatogram of the α enzyme in Fig. 2). From the technical aspect the chromatography appeared to be entirely satisfactory: the enzyme had a finite partition coefficient and there was no question of an abrupt change of the coefficient from ∞ to 0 with change of pH and salt concentration such as Tiselius (1954) had suggested on theoretical grounds. The separations on ion-exchange columns are primarily, though not entirely, dependent on charge differences but attempts to make use of other properties such as solubility, in salt fractionation and liquid–liquid chromatography, were unsuccessful.

There seems little doubt that cathepsin D is the principal proteolytic enzyme of ox spleen as judged by the haemoglobin assay. More than 90% of the activity of a spleen mince could be extracted and about two-thirds of this activity was characterized as cathepsin D. In ox kidney, the proteolytic activity is only about two-thirds of that of the spleen, but again most of the activity is attributable to an enzyme which resembles cathepsin D closely and which may be identical with it (Cebra & Press, unpublished work). The kidney enzyme shows the same complexity as that of the spleen, making it improbable that this multiplicity of form is related to immunological function, since the kidney takes no part in antibody production. Cathepsins B and C account for some of the remaining activity but the presence of other uncharacterized proteolytic enzymes cannot be excluded, though in our work conclusive evidence for the existence of cathepsin A could not be obtained. It is probable that cathepsin D is identical with the enzyme partially purified by Anson (1939). Calculation of the relation of our different enzyme units suggests that the preparation which he obtained was already one-third pure. Cathepsin D may also be related to an enzyme obtained from ox lung (Dannenberg & Smith, 1955), which has a specificity similar to pepsin on the B chain of oxidized insulin but which will not hydrolyse peptide substrates for pepsin.

The low pH optimum on protein substrates (pH 3·0 for haemoglobin and pH 4·2 for serum albumin) is in agreement with the results of previous work on the proteases of the spleen (Anson, 1939), and in other tissues such as pituitary (Adams & Smith, 1951), brain (Kies & Schwimmer, 1942) and erythrocyte stroma (Morrison & Neurath, 1953). However, in both pituitary and stroma a second protease with an optimum at pH 7·0–8·0 was also found and, in one of the earliest reports on the spleen proteases (Hedin, 1904), it was stated that hydrolysis of fibrinogen could be demonstrated in both acetic acid and sodium carbonate solutions. We have been unable to find any significant activity with the haemoglobin assay, at neutral or alkaline pH, whatever method of extraction was used. The pH–activity curves of crude extracts followed closely those of the purified enzymes. A similar low pH optimum has been reported for another spleen enzyme, deoxyribonuclease (Koerner & Sinsheimer, 1957). As the cytoplasmic pH of cells such as monocytes and neutrophils lies in the range pH 4·7–5·5 (Sprick, 1956), and there is a possibility of local variation of pH in different cell structures, it seems probable that these enzymes may function near to their optimum pH.

The most surprising finding has been the large number of different forms in which cathepsin D has been isolated. The reproducibility of the results and the correlation between the quite different techniques of chromatography and electrophoresis in starch gel suggests that there is a genuine difference between the various enzymes. As the enzymes appear to be identical except for their charge at about pH 5·5 and about pH 8·4 the difference would be explicable by the presence of varying amounts of weak acidic and basic groups. For example, the α, β, γ and δ groups of enzymes may contain differing amounts of carboxylic or phenolic groups which are ionized above pH 8·0 but which are unionized at pH 5·5. Similarly, β_1, β_2 and β_3 enzymes may contain different amounts of a weak base such as an imidazole group which is ionized below pH 6·0 but unionized above pH 8·0.

Several possible explanations of this multiplicity of forms were considered. There is a marked variation in the total proteolytic activity of different spleens and in the relative content of the different forms of cathepsin D. Confusion due to the presence of varying amounts of extraneous material, such as blood, which cannot easily be washed out of a spleen, was eliminated by finding that the proteolytic activity of volumes of whole ox blood similar to the volume of the spleen was negligible. More likely was the production of different active forms by limited autolysis during extraction and purification. To test this, one spleen was divided into two portions immediately after mincing. One part was prepared as rapidly as possible and the other was allowed to stand at 2° for 1 week at

pH 4·6, when it was prepared independently. Figs. 1*a* and *b* show that the chromatograms on a DEAE column are identical within the limits of the method. In another experiment, one part of a spleen was prepared directly and another converted into an acetone-dried powder and stored at 2° before extraction. Again the same qualitative and quantitative results were obtained. Further, incubation of partially purified enzymes under a variety of conditions did not affect their chromatographic behaviour on DEAE and CM columns. If autolysis is responsible for the complexity found then it must occur very rapidly and not go beyond a predefined limit in each spleen. This seems improbable.

Another possible explanation is that a single enzyme protein was associated with differing amounts of an impurity or with differing impurities. However, the final products behaved as single components as judged by chromatography and starch-gel electrophoresis under different conditions. They appeared to be monodisperse in the ultracentrifuge and had only one *N*-terminal amino acid in approximately molar ratio, and all had the same specific enzymic activity. This would not exclude the possibility that the differences were due to small charged molecules bound to the protein but, if so, the binding must be exceptionally firm, as it was unaffected by different purification procedures, including ion-exchange chromatography, which would be expected to remove them. Specific binding of small molecules as firmly as this would itself probably imply structural differences between the different forms.

It seems probable therefore that cathepsin D does exist in ox spleen (and probably in ox kidney) in at least ten different molecular forms. Similar findings with other enzymes have been reported (see Markert & Møller, 1959 for references and also Aqvist & Anfinsen, 1959) and it has been suggested, from the work with haptoglobins (Smithies & Walker, 1955) and haemoglobin (Itano, 1957), that this complexity is a reflexion of genetic variations in individual animals. It remains to be investigated whether this is true of cathepsin D.

SUMMARY

1. A proteolytic enzyme has been isolated from bovine spleen, which accounts for two-thirds of the total proteolytic activity of the crude mince. It is present in at least ten forms which differ from one to another in their charge at pH 8·4 or 5·5.

2. The specific activity of the different forms of the enzyme is the same and their purity has been established by starch-gel electrophoresis at pH 9·1 and 5·5, by ultracentrifugal analysis and determination of *N*-terminal amino acid, which is found to be glycine.

3. Cathepsin D has a pH optimum of 3·0 with acid-denatured haemoglobin and 4·2 with acid-denatured albumin. It will not hydrolyse the synthetic substrates described by Fruton (1957–58) for cathepsins A, B and C nor will it hydrolyse the pepsin substrates *N*-acetyl-DL-phenylalanyl-L-diiodotyrosine, L-cysteinyl-L-tyrosine or L-tyrosyl-L-cysteine. The specificity of action on the B chain of oxidized insulin is similar to that of pepsin but more restricted. No difference in specificity could be detected between the different forms of the enzyme.

4. The proteolytic activity of cathepsin D is not affected by cysteine, iodoacetamide, *p*-chloromercuribenzoate, ethylenediaminetetra-acetate or di-*iso*propyl phosphorofluoridate.

5. Cathepsin D is heat-labile and activity is rapidly lost below pH 2·5 at room temperature.

We wish to thank Dr P. A. Charlwood for determining the sedimentation constant and molecular weight of cathepsin D, also Dr R. Pitt-Rivers for giving the three synthetic substrates for pepsin, and Mr A. Hemmings for synthesis of benzoyl-L-arginine amide and sodium monochloromethane sulphonate. We also wish to thank Mr A. Allen for technical assistance.

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Acylative Decarboxylation with Particular Reference to the Acetylative Decarboxylation of the 2:4-Dinitrophenyl Derivatives of some Amino Acids

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In studies on glycine metabolism in normal human subjects (Watts & Crawhall, 1959) and in patients with hyperoxaluria (Watts, Scowen & Crawhall, 1958) we needed to degrade 2:4-dinitrophenylglycine to yield the ^{13}C-labelled carboxyl-group carbon atom selectively as carbon dioxide for mass spectrometric analysis. We attempted to achieve this by oxidative means but were unable to limit the reaction to the carboxyl group. However, base-catalysed acetylative decarboxylation was satisfactory, and the present paper describes the procedure together with the result of an attempt to identify the reaction product other than carbon dioxide. The application of this reaction to the decarboxylation of hippuric acid and of some other dinitrophenyl-amino acids has also been studied. A small amount of carbon dioxide is formed when acetic anhydride and a basic catalyst are heated together in the absence of any other reactant, and we have investigated the time course of this reaction and the relative amounts of carbon dioxide which are evolved when different catalysts are used in order to reduce this to a minimum. The apparent acetylative decarboxylation of the dinitrophenyl derivatives of secondary amino acids is of some theoretical interest, for it is difficult to see how such compounds can form the intramolecular ring structures which have been postulated for the intermediates in this type of reaction (see, for example, Cornforth & Elliott, 1950). The rates of carbon dioxide evolution from dinitrophenylsarcosine and dinitrophenylglycine are not appreciably different and the possible significance of this observation will be discussed.

The acetylative decarboxylation reactions which we have observed may be considered to have an analogy with the biological synthesis of δ-aminolaevulic acid from glycine and succinyl-coenzyme A (cf. Gibson, Laver & Neuberger, 1958; and Kikuchi, Kumar, Talmage & Shemin, 1958). We therefore sought evidence for the succinylative decarboxylation of dinitrophenylglycine and of the related compound glyoxylic acid-2:4-dinitrophenylhydrazone. The latter reaction was of some further interest in view of the possibility of reducing the theoretical product (4-oxoglutaraldehyde-2:4-dinitrophenylhydrazone) to δ-aminolaevulic acid.

EXPERIMENTAL

Melting points were determined by the standard method, except where otherwise stated, and are uncorrected. Micro-analyses were performed by Weiler and Strauss, Oxford. [1-^{13}C]Glycine was synthesized as described previously (Watts & Crawhall, 1959).

Dinitrophenyl derivatives. The dinitrophenyl (DNP) derivatives of alanine, serine, cystine, phenylalanine, tyrosine, leucine and valine were a generous gift from Dr D. F. Elliott. DNP-sarcosine, DNP-proline, DNP-α-amino*iso*butyric acid, DNP-aspartic acid and DNP-[1-^{13}C]glycine were prepared by dissolving the amino acid in

a saturated aqueous solution of sodium hydrogen carbonate and shaking with an excess of 1 fluoro-2:4-dinitrobenzene in methanol for 24 hr. at room temperature. DNP-sarcosine was crystallized from ethyl acetate–*cyclo*hexane (1:9), m.p. 177° (Dalgliesh, Johnson, Todd & Vining, 1950, give m.p. 178°). DNP-proline crystallized from ethyl acetate–*cyclo*hexane (1:9) had m.p. 186° (Dalgliesh *et al.* give m.p. 189°). DNP-α-amino*iso*butyric acid was crystallized from ethyl acetate–*cyclo*hexane (1:9) and acetone–light petroleum (b.p. 40–60°), m.p. 173° (Found: C, 44·0; H, 4·1; N, 15·6. $C_{10}H_{11}O_6N_3$ requires C, 44·6; H, 4·1; N, 15·6%). DNP-δ-aminolaevulic acid was prepared in the same way as the other DNP-amino acids except that 0·25M-sodium phosphate buffer (pH 7·0) was substituted for the saturated sodium hydrogen carbonate solution. DNP-δ-aminolaevulic acid was crystallized from ethyl acetate–*cyclo*hexane (1:9) and had m.p. 156° (decomp.) (Found: C, 45·1; H, 4·0; N, 13·1. $C_{11}H_{11}O_7N_3$ requires C, 44·4; H, 3·7; N, 14·1%).

Acetylative decarboxylation of dinitrophenyl-[1-^{13}C]glycine and purification of the evolved $^{13}CO_2$ for mass-spectrometric analysis. DNP-[1-^{13}C]glycine (approx. 15 mg.; 0·063 m-mole) on a watch glass was dissolved in an equivalent amount of 0·1N-NaOH and the water removed by evaporation *in vacuo*. The dry sodium salt was transferred to a pear-shaped flask (10 ml.) and suspended in redistilled acetic anhydride (1 ml., 10·7 m-moles). Dry helium was passed down a tube to the bottom of the flask, which was connected by a small reflux condenser to a vacuum manifold line. The gas was passed through the flask and condenser and out to the atmosphere by an additional tap *A* for 10 min. before heat was applied to the acetic anhydride. Then tap *A* was closed and the tap connecting the vacuum line to the reaction flask was gently opened. This tap had a groove cut in the barrel (Glascock, 1954) to enable a gentle flow of gas (20 ml./min. at atmospheric pressure) to be maintained. The flask was then heated so that the acetic anhydride refluxed gently. The helium and evolved CO_2 were drawn through three spiral traps (1, 2 and 3) which were cooled in liquid O_2. The reaction mixture changed from a deep yellow to a straw colour and finally became dark brown, with the formation of a small white precipitate. After 0·5 hr. the grooved tap was closed and the pressure in the manifold line reduced to 0·005 mm. Hg. Tap *B*, between the first and second of the liquid O_2-cooled traps, was then closed, as was the main tap connecting the vacuum line to the pumping system (two-stage rotary oil pump, model no. 2SC20, Edwards and Co. Ltd., Crawley, Sussex). The liquid O_2 surrounding the first trap was then replaced by a solid CO_2–ether mixture. Tap *B* was opened for 2 min. and CO_2 allowed to pass from trap 1 to traps 2 and 3. This procedure was designed to separate the CO_2 from any acetic anhydride that had passed over from the reaction vessel into trap 1. The liquid O_2 surrounding traps 2 and 3 was removed and the CO_2 transferred to the cooling limb of a manometer. The tap of the manometer was closed, the CO_2 allowed to vaporize and its volume calculated from the observed pressure change. The pressure in the manometer was again reduced to 0·005 mm. Hg by immersing its cooling limb in liquid O_2; the Dewar flask containing liquid O_2 was replaced by one containing a solid CO_2–ether mixture and the gas transferred to an evacuated flask (250 ml.) which was also fitted with a cooling limb and containing a copper coil (22 guage, 6 ft. coiled on ¼ in. former). The coil was raised to dull-red heat for 10 sec. by the current from a 12v accummulator. The gas was finally transferred to the mass spectrometer (Consolidated Nier Mass Spectrometer model 21–201) and the ^{13}C-abundance measured.

Isolation of the product other than carbon dioxide from the acetylative decarboxylation of dinitrophenylglycine. DNP-glycine (150 mg., 0·62 m-mole) was converted into its anhydrous sodium salt, which was heated under reflux with redistilled acetic anhydride (10 ml., 107 m-moles) for 1·5 hr. The acetic anhydride was removed by steam-distillation and a yellow crystalline solid (77 mg.) separated from the cooled aqueous solution. This was recrystallized from water. The melting point of this derivative could not be satisfactorily determined because it sublimed at 250° in the hot-stage melting-point apparatus, and this may be why the microanalyses have been unsatisfactory (Found: C, 42·7; H, 3·5; N, 16·6. $C_9H_9O_6N_3$ requires C, 42·5; H, 3·5; N, 16·5%. The expected DNP-aminoacetone, $C_9H_9O_5N_3$, requires C, 45·2; H, 3·7; N, 17·6%). The infrared-absorption spectrum was determined and showed a strong peak at 1720 Å suggestive of a carbonyl group, but after attempts to make a semicarbazone the reactants were recovered unchanged.

Acetylative decarboxylation of other dinitrophenylamino acids and of N-dimethylglycine. The reaction was carried out essentially as described for DNP-glycine. The yield of CO_2 was calculated from the volume of gas which was present in the manometer before the hot-copper-wire treatment.

Acetylative decarboxylation of [1-^{13}C]hippuric acid. Hippuric acid was dissolved in 1·5 mol.prop. of 0·1N-NaOH and the reaction continued as described for DNP-glycine.

Comparison of the rates of acetylative decarboxylation of dinitrophenylglycine and dinitrophenylsarcosine. The appropriate DNP derivative (0·5 m-mole) on a watch glass was dissolved in 2·5 ml. of 0·2N-NaOH, and the water removed by evaporation *in vacuo*. The dry residue was then transferred to a pear-shaped flask (25 ml.) and redistilled acetic anhydride (10 ml., 107 m-moles) was added. The flask was fitted with an inlet for passing helium beneath the surface of the acetic anhydride. After helium had been passed through the apparatus for about 10 min. the acetic anhydride was brought to the boil. The effluent gas flowed through a small water-cooled reflux condenser, then passed a cold-finger cooled with solid CO_2–ether and finally via a three-way tap through two traps arranged in series and containing aq. $Ba(OH)_2$ (0·05N, 10 ml. under light petroleum, b.p. 100–120°), the second of these was isolated from the atmosphere by a soda-lime guard tube. The other limb of the three-way tap was connected to a second identical set of traps with aq. $Ba(OH)_2$. Every 10 min. the flow of gas was changed from one set of traps to the other. The contents of the first set of traps were titrated with 0·1N-HCl (with phenolphthalein as indicator). The traps were then washed, refilled and replaced ready for use at the end of the subsequent 10 min. period.

Evolution of carbon dioxide from acetic anhydride and basic catalysts. These experiments were carried out in the apparatus described in the previous experiment. Acetic anhydride (10 ml., 107 m-moles) was heated under reflux with anhydrous sodium acetate (250 mg., 3·05 m-moles), pyridine (0·5 ml., 6·2 m-moles) or β-picoline (0·5 ml., 5·2 m-moles), the CO_2 being collected in aq. $Ba(OH)_2$ and determined titrimetrically.

Evolution of carbon dioxide from succinic anhydride containing sodium succinate as catalyst. The apparatus used was the same as that employed to study the rate of CO_2 evolution during the acetylative decarboxylation of DNP-glycine and DNP-sarcosine, except that the solid CO_2–ether-cooled cold-finger was omitted. Succinic anhydride (50 mg., 0·5 m-mole) was dissolved in 0·5 ml. of 0·1 N-NaOH and the water removed by evaporation *in vacuo*. The dry sodium salt was then mixed with more succinic anhydride (1 g., 10 m-moles) and transferred to the reaction flask, which was heated in an oil bath at 160°. Evolution of CO_2 commenced after 15 min. After 1 hr. the mixture began to darken and after 2 hr. it was jet black.

Reaction of succinic anhydride with dinitrophenylglycine. (*a*) DNP-glycine (116 mg., 0·48 m-mole) was converted into its sodium salt, mixed with succinic anhydride and heated to 160° as in the previous experiment. At the end of 3 hr. the reaction mixture, which had become jet black, was extracted with hot water (10 ml.) and the water-insoluble residue was dissolved in ethanol (40 ml.). Samples from both solutions were submitted to chromatography on buffered Whatman no. 1 paper with the 2-methylbutan-2-ol (*tert.*-pentanol) system of Blackburn & Lowther (1951). The water-insoluble material gave a faint yellow spot (R_F 0·36). No yellow derivatives were present in the water-soluble material. Control spots of DNP-glycine and DNP-δ-aminolaevulic acid had R_F values of 0·38 and 0·63 respectively.

(*b*) Four samples of DNP-glycine (12 mg., 0·05 m-mole) were converted into the sodium salt. Succinic anhydride (100 mg.) was added to each and the mixture stirred. They were then transferred to an oil bath at 160° and removed from the bath after 15, 30, 45 and 60 min. respectively. The mixtures were then extracted with hot water (10 ml. each) and the insoluble residues, 7·2, 9·0, 7·8 and 5·8 mg. respectively, were dissolved in acetone. Samples of the four aqueous and four acetone solutions were submitted to chromatography as described in (*a*) above.

The aqueous solutions showed only DNP-glycine in quantities which decreased with increasing time of heating. The acetone-soluble material showed a brown spot at the origin and a trace of DNP-glycine. No spot corresponding to the marker DNP-δ-aminolaevulic acid (R_F 0·70) was observed.

Reaction of succinic anhydride with glyoxylic acid-2:4-dinitrophenylhydrazone. (*a*) Glyoxylic acid-2:4-dinitrophenylhydrazone (127 mg., 0·5 m-mole) was converted into its sodium salt and mixed with succinic anhydride (1 g.). The mixture was heated in an oil bath to 160° and the rate of evolution of CO_2 studied as described above.

(*b*) Glyoxylic acid-2:4-dinitrophenylhydrazone (46·7 mg., 0·18 m-mole) was converted into its sodium salt, mixed with powdered succinic anhydride (300 mg.) and heated at 160° for 1 hr. The mixture was cooled, suspended in 5 ml. of hot water and then cooled to 2°. The precipitate was removed by filtration (31·8 mg.). Chromatography of this product in butan-1-ol–ethanol–aq. 0·5 N-NH_3 soln. (70:10:20, by vol.) showed three yellow spots, R_F values 0·59, 0·76 and 0·86. Catalytic reduction of the product at room temperature and atmospheric pressure in water with PtO as catalyst gave a colourless solution which rapidly turned green on exposure to air. (A control reduction of the 2:4-dinitrophenylhydrazone of glyoxylic acid showed that 7 mol.prop. of hydrogen was absorbed.) No free δ-aminolaevulic acid could be detected after chromatography in butan-1-ol–acetic acid–water (60:15:25, by vol.) of the reduced solution with the reagents described by Shuster (1956).

RESULTS

In our first attempts to degrade DNP-[1-^{13}C]-glycine by acetylative decarboxylation we heated 15 mg. of the amino acid derivative with 4 ml. of acetic anhydride and 100 mg. of anhydrous sodium acetate, and used nitrogen as the carrier gas. The carbon dioxide was collected in spiral traps which were cooled in liquid oxygen, and its volume was measured manometrically before it was transferred to the mass spectrometer without any further purification. This procedure yielded approximately 100 % of the theoretical volume of carbon dioxide, but the values which were obtained for the isotope enrichment of replicate samples of known ^{13}C-abundance did not agree with one another, and were too low. If the carbon dioxide was converted into barium carbonate and reliberated with sulphuric acid, approximately 100 % of the theoretical

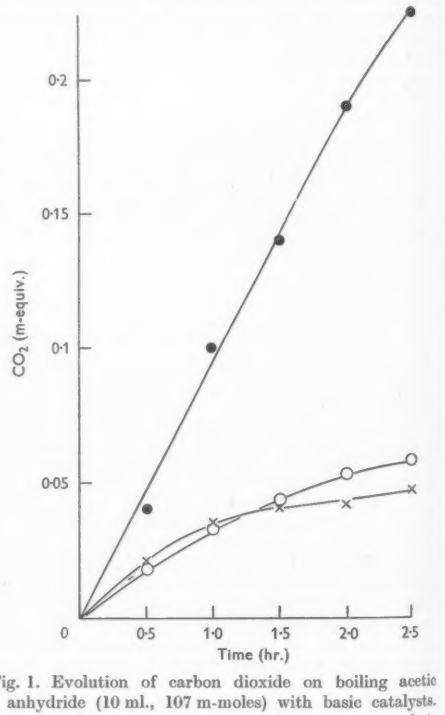

Fig. 1. Evolution of carbon dioxide on boiling acetic anhydride (10 ml., 107 m-moles) with basic catalysts. ●, Anhydrous sodium acetate (250 mg., 3·05 m-moles); ○, β-picoline (0·5 ml., 5·2 m-moles); ×, pyridine (0·5 ml., 6·2 m-moles).

volume of carbon dioxide was still obtained, but the results of the ^{13}C-determination although reproducible were still approximately 10% too low. The observation that the amount of carbon dioxide which was obtained from DNP-alanine under these circumstances was equivalent to 110% of the theoretical value suggested that the apparent 100% yield of carbon dioxide which we had obtained from DNP-glycine might have been due to the formation of extraneous carbon dioxide by the reagents. It was shown that anhydrous sodium acetate, pyridine and β-picoline all caused the evolution of some carbon dioxide when boiled with acetic anhydride and this occurred most extensively with anhydrous sodium acetate (Fig. 1). However, β-picoline and pyridine appeared to be less efficient catalysts than anhydrous sodium acetate for the acetylative decarboxylation of DNP-glycine, because, although DNP-alanine yielded 90% of the theoretical volume of carbon dioxide when these reagents were used, the yield from DNP-glycine was only 60%. In order to utilize the greater catalytic potency of sodium acetate without introducing more than the minimum amount of extraneous carbon dioxide from the reagents, we prepared the anhydrous sodium salts of the DNP-amino acids and treated these with the smallest convenient volume (1 ml.) of acetic anhydride. A further trace of extraneous carbon dioxide was eliminated by using pure helium (supplied for anaesthetic purposes) instead of 'industrial' nitrogen as the inert carrier gas. The yield of carbon dioxide under these conditions was reproducible, but if the barium carbonate step was omitted, the apparent ^{13}C-enrichment of DNP-[1-^{13}C]glycine prepared from [1-^{13}C]glycine of known isotope content was still variable, and it appeared that there might be some impurity in the gas which was removed when the carbon dioxide was precipitated as barium carbonate. Purification of the gas in this way doubled the length of time required for each analysis and was very difficult to carry out on such a small scale without the ingress of traces of atmospheric carbon dioxide. The carbon dioxide which was obtained from the acetylative decarboxylation of DNP-amino acids, and which had not been purified by precipitation as barium carbonate, yielded an ion of mass 43 in the mass spectrometer as well as the ions of masses 44 and 45 due to $^{12}CO_2$ and $^{13}CO_2$ respectively. The peak at mass 43 was variable in height but was sometimes as great as that at mass 45 due to $^{13}CO_2$. When the gas was submitted to the barium carbonate treatment, the peak at mass 43 disappeared. The nature of the impurity responsible for the production of an ion of mass 43, and the reason for its apparent interference, is unknown. The free radical ($^{12}CH_3 \cdot {}^{12}CO \cdot$) is an obvious possible impurity of mass 43 and might have been formed in the ionizing beam of the mass spectrometer from, for example, acetone or acetic anhydride. However, when the gas was purified by allowing the previously frozen gas to evaporate from a container cooled in solid carbon dioxide–ether, the peak due to this ion did not disappear though it sometimes decreased. The other anomaly is that the height of the mass 43 peak was never more than 1% of that at mass 44 (due to $^{12}CO_2$) so that the isotope enrichment might have been expected to have been decreased by 1% at the most if this peak was interfering, but in fact the apparent isotope enrichment was always increased when it was present, suggesting that some additional impurity of mass 45 was also present. This could not be confirmed because a peak of mass 45 is always present in carbon dioxide owing to the natural abundance of the ^{13}C isotope. Nevertheless, it was found empirically that this impurity or its precursor could be removed by exposing the gas to a copper wire electrically heated to dull-red heat for 10 sec., and it is presumed that pyrolysis of the impurity took place as after this treatment no peak of mass 43 was detectable. The exact quantity of extraneous carbon dioxide evolved by the reagents cannot be assessed directly because the catalytic activity of the sodium salt of DNP-glycine cannot be determined, but it was found that under the conditions which we describe the isotopic enrichment of the carbon dioxide obtained was always 6% less than that expected from the known isotopic enrichment of ^{13}C in the glycine from which the DNP-glycine was prepared (Table 1), and this correction is always applied in routine analyses.

Various other DNP-amino acids were submitted to this reaction (Table 2). The DNP derivatives of aliphatic amino acids other than glycine gave yields of carbon dioxide between 85 and 95%. The decarboxylation of DNP-serine was not very satisfactory, possibly because the elements of water can be removed, leaving an unsaturated derivative which can undergo alternative side reactions. DNP-aspartic acid yielded more than 100% of the maximum possible yield of carbon dioxide, and

Table 1. *Isotope enrichment of carbon dioxide obtained by combustion of* [1-^{13}C]*glycine and by acetylative decarboxylation of dinitrophenyl-*[1-^{13}C]*-glycine and* [1-^{13}C]*hippuric acid made from it*

Substance	Observed isotope enrichment in sample (atoms % excess)	Isotope enrichment after correction for dilution (atoms % excess)
[1-^{13}C]Glycine	0·548±1%	49·8
DNP-[1-^{13}C]glycine	1·029±1%	46·7
[1-^{13}C]Hippuric acid	1·026±1%	46·3

some decarboxylation from the β-carboxyl group may have taken place. The sodium salt of DNP-tyrosine was only sparingly soluble in water, so the lower yield of carbon dioxide may have been caused by incomplete conversion of the acid into its sodium salt. The yield of carbon dioxide from the sodium salt of hippuric acid was increased from 35 to 50% of the theoretical volume by the use of 1·5 instead of 1·0 mol.prop. of sodium hydroxide. The isotopic enrichment of the carbon dioxide obtained from [1-^{13}C]hippuric acid by this method was also 6% less than the theoretical value, and with the application of a suitable correction factor, isotopic analyses of [1-^{13}C]hippuric acid could be carried out as a routine procedure.

The rate of evolution of carbon dioxide by the action of acetic anhydride on the sodium salts of DNP-glycine and DNP-sarcosine were compared under identical conditions, and the results (Fig. 2) indicated that carbon dioxide was evolved at similar rates from the two substances.

An investigation was also made into the possibility of using succinic anhydride as a reagent for the acylative decarboxylation of DNP-glycine because of the similarity of such an acylation to the biological synthesis of δ-aminolaevulic acid. The results of these experiments are rather difficult to assess because the action of heat on succinic anhydride containing sodium succinate alone yielded an amount of carbon dioxide equivalent to 40% of that to be expected if an equivalent quantity of DNP-glycine sodium salt had been present (Fig. 3). When succinic anhydride was treated with the sodium salt of DNP-glycine a yield of 115% of the expected amount of carbon dioxide was obtained. No isotopic label was used in these experiments so it was not known how much of this

Table 2. *Yields of carbon dioxide obtained from the acetylative decarboxylation of some dinitrophenyl-amino acids and related compounds*

	Yield of CO_2 (%)
DNP-glycine	80
DNP-alanine	95
DNP-leucine	87
DNP-valine	97
DNP-serine	40
DNP-cystine	84
DNP-aspartic acid	120
DNP-tyrosine	64
DNP-phenylalanine	77
DNP-sarcosine	80
DNP-proline	95
N-Dimethylglycine	86
DNP-α-amino*iso*butyric acid	10
Hippuric acid	50

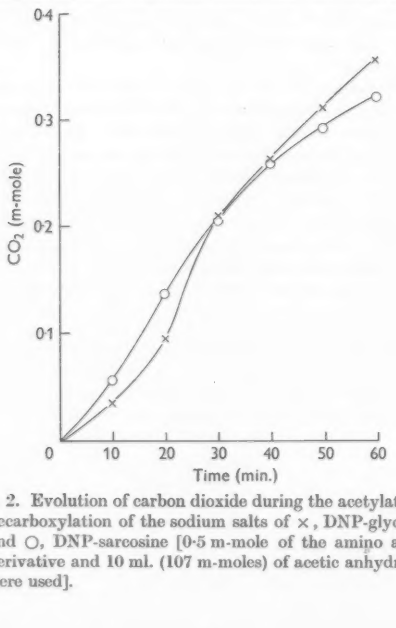

Fig. 2. Evolution of carbon dioxide during the acetylative decarboxylation of the sodium salts of ×, DNP-glycine and ○, DNP-sarcosine [0·5 m-mole of the amino acid derivative and 10 ml. (107 m-moles) of acetic anhydride were used].

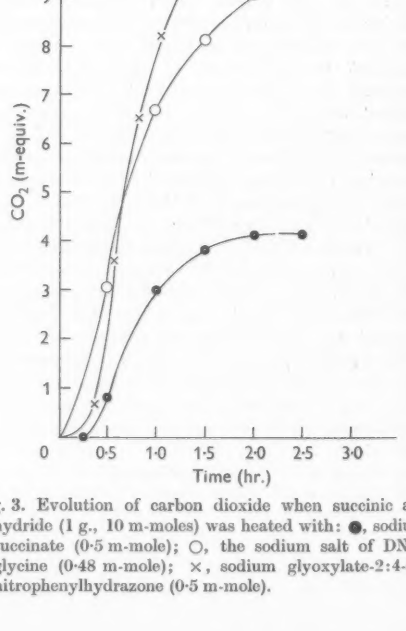

Fig. 3. Evolution of carbon dioxide when succinic anhydride (1 g., 10 m-moles) was heated with: ●, sodium succinate (0·5 m-mole); ○, the sodium salt of DNP-glycine (0·48 m-mole); ×, sodium glyoxylate-2:4-dinitrophenylhydrazone (0·5 m-mole).

carbon dioxide came from the DNP-glycine and how much from the succinic anhydride, but the results did suggest that decarboxylation of the DNP-glycine was taking place.

In another series of experiments in which the time of heating of the reactants was varied, chromatographic analysis of the products showed that the amount of free DNP-glycine decreased as the length of heating increased but it was not replaced by any other DNP derivative. In an attempt to synthesize δ-aminolaevulic acid by the succinylation procedure, succinic anhydride was treated with the sodium salt of the 2:4-dinitrophenylhydrazone of glyoxylic acid. Carbon dioxide was generated in this reaction (Fig. 3), but if the reaction was continued for 3 hr. the mixture became black and no 2:4-dinitrophenylhydrazone could be identified in the product. At the end of 1 hr. it could be shown by paper chromatography that at least three 2:4-dinitrophenylhydrazones were present. If one of these had been the expected 2:4-dinitrophenylhydrazone of 4-oxoglutaraldehyde, reduction of the mixed products should have given δ-aminolaevulic acid (Meister & Abenschein, 1956; Smith, 1958). The electrolytic-reduction method of Smith (1958) rapidly destroyed free δ-aminolaevulic acid. By the method of Meister & Abenschein (1956) no δ-aminolaevulic acid could be identified chromatographically.

DISCUSSION

The reaction of an amino acid with acetic anhydride in the presence of anhydrous sodium acetate as a catalyst to yield an acylamido ketone and carbon dioxide was first described by Dakin & West (1928), and since then many workers have investigated this reaction and some have studied the rate and quantity of carbon dioxide evolution. It is therefore surprising that the original observation of Perkin (1886) that anhydrous sodium acetate and acetic anhydride evolve carbon dioxide on heating has only recently received any attention. Rondestvedt, Manning & Tabibian (1950) carried out the Dakin & West reaction on [1-^{14}C]alanine and obtained a lower proportion of $^{14}CO_2$ in the evolved gas than they had expected, but they attributed this to an ingress of atmospheric carbon dioxide. Fahey (1957) investigated the reaction of [1-^{14}C]-acetic anhydride on *o*-chlorophenylacetic acid in the presence of anhydrous sodium acetate and found that three reactions can occur simultaneously. The main reaction is the acetylative decarboxylation of *o*-chlorophenylacetic acid with the formation of *o*-chlorophenylacetone. A very minor reaction is that of the mixed anhydride of *o*-chlorophenylacetic acid and acetic acid with [1-^{14}C]acetic anhydride, with the formation of *o*-chlorophenylacetone and $^{14}CO_2$ derived from the carbonyl group of acetic anhydride. A third reaction is the intermolecular reaction of acetic anhydride molecules with the evolution of $^{14}CO_2$. Results which we have obtained (Fig. 2) also demonstrate that carbon dioxide is evolved when acetic anhydride is heated with various basic catalysts. In order to reduce the amount of extraneous carbon dioxide which is formed to a minimum, we have converted the DNP-glycine into its anhydrous sodium salt. Even under these conditions it appears that carbon dioxide is evolved from the reagents because the mass-spectrometric analysis of the evolved carbon dioxide derived from DNP-[1-^{13}C]glycine is 6% lower than the theoretical value (Table 1). This additional carbon dioxide may have arisen from either the second or third reaction described by Fahey (1957).

Wiley (1950), Cornforth & Elliott (1950) and Buchanan, Reid, Thomson & Wood (1957) have discussed the possibility of formation of cyclic intermediates when secondary amino acids are submitted to the Dakin & West reaction. Wiley (1950) suggested that decarboxylation can precede acetylation in the reaction of acetic anhydride with sarcosine. We have found that DNP-aliphatic amino acids, with the exception of DNP-α-amino-*iso*butyric acid, which possesses no free α-hydrogen atom, readily decarboxylate under our conditions. If decarboxylation preceded acetylation it is difficult to see why DNP-α-amino*iso*butyric acid should not decarboxylate as readily as DNP-alanine unless considerable steric hindrance from the α-methyl group is postulated. The fact that a small quantity of carbon dioxide is evolved (Table 2) can be explained by Fahey's (1957) second type of reaction, that is that the mixed anhydride of the DNP-amino acid and acetic acid can react with the reagents with the formation of carbon dioxide. As this DNP-amino acid does not undergo acylative decarboxylation, it will remain for a longer period in the reaction mixture, enabling this second type of reaction to occur to a greater extent. Buchanan *et al.* (1957) found that *N*-phenylglycine and *N*-phenyl-*N*-acetylglycine were decarboxylated under the Dakin & West (1928) conditions, but that *N*-phenyl-*N*-formylglycine was not. On the basis of this observation they questioned whether the current theories are adequate to explain this result, although we are not aware of any experimental evidence that *N*-formyl amino acids can form oxazolones.

DNP-sarcosine readily yields carbon dioxide on treatment of its sodium salt with acetic anhydride and the rate of evolution was similar to that observed with DNP-glycine (Fig. 2 and Table 2). It is difficult to see how any intramolecular cyclization can take place with DNP-sarcosine because it

possesses a tertiary nitrogen atom. It can therefore be deduced from the results with DNP-sarcosine and DNP-proline that the formation of an intramolecular cyclic structure is not obligatory for the acylative decarboxylation of all substituted amino acids. It is possible that the presence of a 2:4-dinitrophenylmethylamino group attached to the α-carbon atom causes sufficient activation to permit attack by acetic anhydride at the α-position and that the reaction is therefore analogous to the acylative decarboxylation of *o*-chlorophenylacetic acid by acetic anhydride (Fahey, 1957). The similarity between the rates of acetylative decarboxylation of DNP-sarcosine and DNP-glycine raises the possibility that this latter reaction may also proceed without the formation of an intramolecular cyclic intermediate.

Kikuchi *et al.* (1958) have shown in a biological system that, in the reaction of succinyl-coenzyme A with glycine in the presence of pyridoxal phosphate and magnesium ions as catalysts, decarboxylation does not precede succinylation, and a similar type of condensation has been postulated to explain the synthesis of aminoacetone by washed suspensions of *Staphylococcus aureus* (Elliott, 1959). It therefore seemed that there might be a similarity between the biological synthesis of δ-aminolaevulic acid and the acylative decarboxylation of DNP-glycine which we have investigated. Succinic anhydride has not previously been described as a reagent for acylative decarboxylation, though Fittig (1897) showed that acetic anhydride reacted with succinic acid to form laevulic acid. We found that heating DNP-glycine and the 2:4-dinitrophenylhydrazone of glyoxylic acid with succinic anhydride and a basic catalyst caused the reactants to evolve carbon dioxide, but a simple succinylative decarboxylation did not take place.

SUMMARY

1. The acetylative decarboxylation of 2:4-dinitrophenylglycine has been investigated with particular reference to the degradation of dinitrophenyl-[1-^{13}C]glycine to yield the carboxyl-group carbon atom in the form of carbon dioxide for mass-spectrometric analysis. An 80–85% yield of carbon dioxide was obtained from the carboxyl group of dinitrophenylglycine and the observed ^{13}C enrichment of this gas has to be multiplied by a factor of 1·06 to allow for a small but constant amount of extraneous carbon dioxide which is formed as a result of unavoidable side reactions. The procedure has been used with minor modifications for the similar degradation of [1-^{13}C]hippuric acid.

2. The yield of carbon dioxide from the acetylative decarboxylation of the dinitrophenyl derivatives of other primary and secondary amino acids has been investigated.

3. The decarboxylation of dinitrophenylsarcosine and dinitrophenylproline indicates that the formation of intramolecular cyclic intermediates may not be universally essential for acetylative decarboxylation of substituted amino acids.

4. Carbon dioxide was evolved when succinic anhydride was heated with the sodium salts of dinitrophenylglycine and the 2:4-dinitrophenylhydrazone of glyoxylic acid but no evidence for the formation of the respective derivatives of δ-aminolaevulic acid was obtained.

5. The experimental findings are discussed with particular reference to the postulated mechanisms of acylative decarboxylation reactions.

We are pleased to acknowledge our indebtedness to Dr E. F. Scowen for his continued encouragement in this work, to Professor A. Wormall, F.R.S., who generously placed the mass spectrometer in his Department at our disposal, to Dr D. F. Elliott and Dr J. J. Scott for gifts of DNP-amino acids and methyl δ-aminolaevulate respectively, to Dr R. K. Callow, F.R.S., for measurement of the infrared spectra, and to Miss E. D. Bell for her skilled assistance.

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Metabolism of Xanthurenic Acid in the Rat

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The chromatographic pattern of metabolites of tryptophan and xanthurenic acid in urine of the normal rat has been described by Baglioni, Fasella, Turano & Siliprandi (1960). Administration of xanthurenic acid caused the excretion of three major components, one of which is xanthurenic acid. When heated at 100° in strongly acidic solutions, the other two compounds yielded xanthurenic acid. Dalgliesh (1952) reported the presence, in the urines of tryptophan-fed pyridoxine-deficient rats, of a compound in which xanthurenic acid is conjugated with sulphate and some other unknown residue. In the urine of xanthurenic acid-treated albino rats, Kotake & Nogami (1954) and Kotake (1957) found a double ethereal sulphate and a glucuronide derivative, and Rothstein & Greenberg (1957) found a diglucuronide–monoserine derivative and a monoglucuronide–monoserine derivative. In the present paper we describe the separation and identification of two metabolites of xanthurenic acid from rat urine.

EXPERIMENTAL

Separation and purification of metabolites. Xanthurenic acid (a kind gift of Professor Musajo, University of Padova) was administered to white albino rats of about 200 g. body wt. in amounts of 300 mg./kg. The urine passed in the 24 hr. after the administration was collected and adsorbed on and eluted from deactivated charcoal according to Dalgliesh (1955). The dry residue of the urinary extract was dissolved in 1 ml. of water; 0·05 ml. was analysed by electrochromatography (Baglioni *et al.* 1960) and 0·3 ml. was applied to the top of a Dowex-50 ion-exchange column (50 × 0·9 cm., 150–200 mesh) in the hydrogen form and eluted with water. The effluent was collected in fractions of 0·5 ml. at a rate of 1·5 ml./hr. The fractions were examined for fluorescence in ultraviolet light (366 mμ) and, after dilution to 3 ml. with water, for their extinction at 280 and 340 mμ. The fractions corresponding to the absorption and fluorescence peaks were collected and analysed by paper chromatography (Dalgliesh, 1956) and electrochromatography (Baglioni *et al.* 1960). Each of the components so separated was further purified by submitting it to electrophoresis on a cellulose column (51 cm. × 4 cm.; sodium acetate buffer, pH 5·4, *I* 0·01; 600 v, 8 mA for 9 hr.). The bottom of the column was connected to the anode. On completion of the electrophoretic run the column was eluted with the same buffer at the rate of 12 ml./hr. and the effluent collected in fractions of 3 ml. each. The effluent was examined for fluorescence and extinction as described above. To remove the buffer used in electrophoresis, the major component was concentrated under high vacuum and rechromatographed on a Dowex-50 column as described above. Fractions containing the purified substance were collected and lyophilized.

Analytical tests. Sulphate ion was detected by precipitation with barium chloride and glucuronic acid by the naphtharesorcinol reaction. Amino acids were detected by paper chromatography in phenol (Consden, Gordon & Martin, 1944), lutidine–collidine–water (Dent, 1948) and 80% pyridine (Block & Bolling, 1951) and by paper electrophoresis at pH 6·0 in 0·02 M-potassium phosphate buffer. Pauly's reaction for phenolic groups was according to Feigl (1956).

RESULTS

The pattern of the electrochromatographic separation of the urinary extracts after administration of xanthurenic acid is shown in Fig. 1. The separation

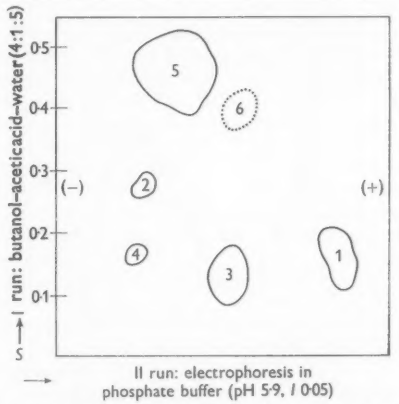

Fig. 1. Electrochromatographic separation of metabolites of xanthurenic acid in rat urine. One-twentieth of the urine eliminated by one rat in the 24 hr. after the administration was applied at the corner of a square paper. The paper was first developed by descending chromatography (upward direction in the figure) and subsequently submitted to electrophoresis (horizontal direction in the figure). Spots 1, 2, 3 and 4 are derivatives of xanthurenic acid, 5 is xanthurenic acid and 6 is urinary indican. Spots 1 and 3 are substances I and II respectively. Spots 1–5 are fluorescent; spot 6 was detected with *p*-dimethylaminobenzaldehyde.

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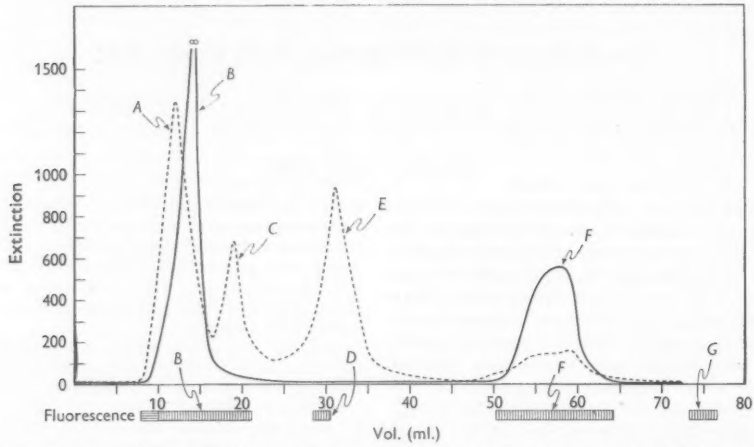


Fig. 2. Separation of metabolites of xanthurenic acid on a Dowex-50 column. About one-third of the urine eliminated by one rat in the 24 hr. after the administration was washed down the column with water at the rate of 1·5 ml./hr. The effluent was collected in fractions of 0·5 ml. each. Extinction was determined after diluting each fraction to 3 ml. with water. ——, at 340 mμ; – – –, at 280 mμ. Fluorescence: ▤, green–yellow; ▥, blue. *A*, Urinary indican; *B*, substance I; *C*, unknown metabolite, present also before administration of xanthurenic acid; *D*, derivative of xanthurenic acid (spot 2 in Fig. 1); *E*, unknown metabolite, present also before administration of xanthurenic acid; *F*, substance II; *G*, derivative of xanthurenic acid (spot 4 in Fig. 1).

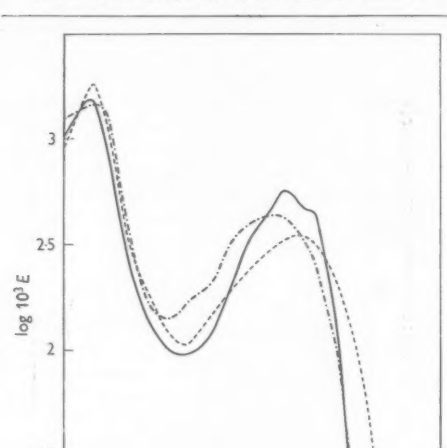


Fig. 3. Absorption spectra of substance I. Solvents: – – –, 0·1 N-HCl; ——, 33 mM-potassium phosphate buffer, pH 7·0; —·—, 0·1 N-NaOH. Solutions used for the determination of the spectrum contained in 3 ml. one-fiftieth of the amount of substance I eliminated by one rat in the 24 hr. after administration of xanthurenic acid.

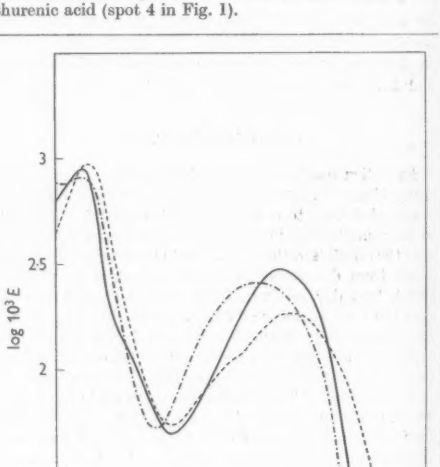


Fig. 4. Absorption spectra of substance II. Solvents: – – –, 0·1 N-HCl; ——, 33 mM-potassium phosphate buffer, pH 7·0; —·—, 0·1 N-NaOH. Solutions used for the determination of the spectrum contained in 3 ml. one-fiftieth of the amount of substance II eliminated by one rat in the 24 hr. after administration of xanthurenic acid.

Table 1. *Properties of substances I and II*

	Substance I	Substance II
Before hydrolysis		
R_F in butanol–acetic acid–water (4:1:5, by vol.)	0·14	0·09
R_F in 20% (w/v) KCl	0·47	0·53
Anodic displacement* (cm.)	25·00	14·50
Pauly's reaction	Delayed	Negative
Ninhydrin test	Negative	Negative
After hydrolysis		
Period of hydrolysis in 5N-HCl at 100° (hr.) ...	3	6
Test for sulphate	Positive	Negative
Naphtharesorcinol test	Negative	Positive
Ninhydrin test	Positive	Positive
Chromatographic test (Dalgliesh, 1956) for xanthurenic acid	Positive	Positive

* In potassium phosphate buffer, pH 5·9, I 0·05; 10v/cm. length of paper; 8 hr.

obtained by the first chromatography on Dowex-50 ion-exchange column is shown in Fig. 2. Fluorescence peaks D and G, which are hardly detectable in the extinction curve because the amounts are small, correspond respectively to spots 2 and 4 of the electrochromatogram. Substances I (peak B) and II (peak F) were separately submitted to electrophoresis on a cellulose column. Substance I was thus separated from urinary indican and from an unidentified substance present in very small amounts and characterized by a green fluorescence; substance II was separated from an unidentified impurity absorbing at 280 mμ. About 6 mg. of substance I and 4 mg. of substance II were obtained from the urine eliminated by one rat in the 24 hr. after the intraperitoneal administration of 60 mg. of xanthurenic acid.

The absorption spectra of purified substances I and II at different pH values are shown in Figs. 3 and 4. The properties of substances I and II are given in Table 1. The ninhydrin-positive substance obtained upon hydrolysis of both compounds had the same R_F values as glycine when chromatographed in phenol and collidine–lutidine. Other known ninhydrin-positive substances with a similar chromatographic behaviour and which could be confused with glycine are glutamic acid and serine. Glutamic acid, however, moves rapidly towards the anode when submitted to paper electrophoresis at pH 6·0, whereas the substance in question does not move at this pH. The R_F value of serine in 80% pyridine is 0·39, whereas that of our substance and of glycine is 0·27. Moreover, in all solvents used our ninhydrin-positive substance could not be separated from authentic samples of glycine, but was easily resolved from authentic samples of serine and glutamic acid; it seems highly probable therefore that the substance in question is glycine.

DISCUSSION

The above results show that substance I is a conjugate of xanthurenic acid with sulphate and glycine and substance II is a conjugate of xanthurenic acid with glucuronic acid and glycine. Some information on the structure of both substances is provided by their absorption spectra and reactivity with ninhydrin and Pauly's reagent. Neither substance I nor substance II reacts with ninhydrin before hydrolysis. The amino group of glycine is therefore involved in the bond, probably with the carboxyl group of xanthurenic acid. Substance II does not react with Pauly's reagent for phenols so that it seems unlikely that the —OH in position 8 of the xanthurenic acid residue is free (the —OH in position 4 is *para* to the nitrogen atom and has feeble phenolic properties, so that direct coupling with Pauly's reagent is unlikely). With substance I Pauly's reaction is positive but greatly delayed; this is compatible with substance I, which is readily hydrolysed, having a labile sulphate group at position 8.

Further evidence for the fact that in both compounds the hydroxyl group in position 8 is bound is provided by the absorption spectra. Neither shows a bathochromic shift at alkaline pH values. According to Ewing & Steck (1946), at alkaline pH 8-hydroxyquinolines show a shift of the absorption peak from 310 mμ to higher wavelengths and this shift does not occur when the hydroxyl group in position 8 is bound. Conjugation is not likely at position 4 since in mammals 4-quinolones do not conjugate directly in position 4 but are first hydroxylated elsewhere, and are excreted as conjugates of hydroxyquinolones (Smith, 1953). This suggests that there is insufficient of the hydroxylated or lactim form present to permit conjugation and that, biologically, quinolones behave as if they were in the keto or lactam form.

The spectra of our substances are in agreement with the above general considerations. In both compounds the shift of the peak from 340 mμ to higher wavelengths at acidic pH values is very slight and is accompanied by a decrease in extinction. There are similar features in the spectrum of 8-methylxanthurenic acid ethyl ester (see Fig. 5), which has structural characteristics similar to those suggested for our compounds (the hydroxyl group in position 8 and the carboxyl group in position 2 are bound, whereas the hydroxyl group in position 4 is free). Compounds in which the hydroxyl group in position 4 is conjugated, e.g. 4-quinolylmethyl ether (see fig. 19 of Ewing & Steck, 1946) show a greater shift of the peak and no decrease of its intensity at acidic pH values. The above considerations suggest that the hydroxyl group in position 4 of the xanthurenic acid residue is free both in substance I and substance II.

It is concluded that substance I is a compound formed by xanthurenic acid, glycine and sulphate. Since the hydroxyl group in position 4 is free, sulphate probably esterifies the hydroxyl group in position 8, whereas glycine is bound by a peptide link to the carboxyl group (see formulae I and II).

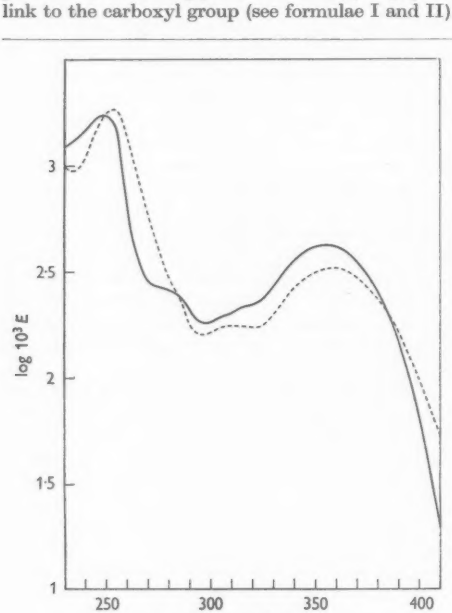
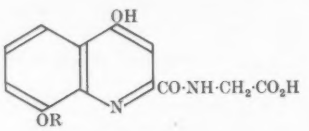


Fig. 5. Absorption spectra of 8-methylxanthurenic acid ethyl ester (46·5 μg. in 3 ml.) in: – – –, 0·1 N-HCl; ——, 33 mM-potassium phosphate buffer, pH 7·0. In 0·1 N-NaOH the substance was too insoluble to yield accurate extinction values, but showed a maximum at 240 mμ and a broad maximum in the region from 320 to 340 mμ.

Substance II is a compound formed by xanthurenic acid, glucuronic acid and glycine. Glycine is bound to the carboxyl group and glucuronic acid is linked to the hydroxyl group in position 8. Apart from any other consideration it seems probable that the latter bond is of glucuronide rather than of glucuronate nature since substance II is much more electronegative than xanthurenic acid (see behaviour in electrophoresis and on Dowex-50), which suggests that it has some free acidic group besides that of xanthurenic acid or glycine.



(I: R = SO_3H)
(II: R = $C_6H_9O_6$)

The chromatographic properties of substances I and II correspond to two compounds found by Dalgliesh (1956) in the urine of tryptophan-fed, pyridoxine-deficient rats and tentatively identified as derivatives of xanthurenic acid. Moreover, Dalgliesh (1952) described a strongly acidic compound very similar in spectrophotometric and chromatographic properties to our substance I. The hydrolysis products of this compound suggested that xanthurenic acid was conjugated with sulphate and some other unknown substance. It seems reasonable to assume that the compound of Dalgliesh is the same as substance I.

In the urines of rats given xanthurenic acid, Kotake & Nogami (1954) and Kotake (1957) reported the presence of a double ethereal sulphate and of a glucuronide derivative, but these authors did not note the presence of an amino acid among the hydrolysis products of either compound. Rothstein & Greenberg (1957) described a diglucuronide–monoserine derivative and a monoglucuronide–monoserine derivative of xanthurenic acid in the urine of rats. In view of the improbability of conjugation in position 4 (Smith, 1953) and our own results more direct evidence is needed for the existence of xanthurenic acid derivatives conjugated in position 4. The conclusions of the Japanese authors might have been affected by the presence of contaminants in their preparations of xanthurenic acid derivatives; the latter were isolated by paper chromatography, a technique which in our hands proved unsuitable for complete purification. The assumption of Rothstein & Greenberg rests on the observation of two glucuronic acid derivatives of xanthurenic acid, one of which can be transformed into the other by mild treatment

with acid; no direct proof, however, is given that this transformation is brought about by detachment of a glucuronic residue from the hydroxyl group in position 4 of xanthurenic acid. The fact that the R_F values of Rothstein & Greenberg's compounds B and D in butanol–acetic acid–water (4:1:5) differ from those of our compounds indicate that they are not the same substances. The difference might tentatively be ascribed to the fact that xanthurenic acid can be conjugated in the living organism in several ways and that differences exist not only between various species, e.g. the rabbit and the rat (Rothstein & Greenberg, 1957), but also between different races of the same species.

SUMMARY

1. Two major metabolites of xanthurenic acid were found in the urine of albino rats after intraperitoneal administration of xanthurenic acid.

2. These metabolites were isolated by chromatography on Dowex-50 and column electrophoresis on cellulose powder.

3. The spectral and chemical properties of the two compounds indicate that one is a derivative of xanthurenic acid in which the hydroxyl group in position 8 is bound to sulphate and the carboxyl group to glycine; the other compound differs in that the hydroxyl group in position 8 is bound to glucuronic acid.

The authors are grateful to Professor Benassi of the Institute of Pharmaceutical Chemistry of the University of Padova for a kind gift of a sample of 8-methylxanthurenic acid ethyl ester.

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The Breakdown of Pyruvate by Cell-Free Extracts of the Rumen Micro-organism LC

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The micro-organism LC, isolated from the sheep rumen by Elsden, Volcani, Gilchrist & Lewis (1956), ferments lactate and carbohydrates with the formation of volatile acids of chain length up to hexanoate. Washed cells break down pyruvate, giving carbon dioxide, acetate and butyrate as the main products (Elsden & Lewis, 1953). Supplementing the pyruvate with acetate, propionate or butyrate results in the formation of large amounts of butyrate, pentanoate or hexanoate respectively. Elsden & Lewis concluded that in LC the higher volatile fatty acids are produced by a mechanism similar to that established in *Clostridium kluyveri* by Barker and his colleagues (Barker, 1957*a*). Whereas the latter organism obtains acetylcoenzyme A for fatty acid synthesis by the anaerobic oxidation of ethanol, it was presumed that in LC this intermediate is derived from pyruvate. Preliminary experiments by S. R. Elsden & C. Warner (unpublished work) indicated that dried preparations catalyse the anaerobic oxidation of pyruvate to acetate and a more detailed investigation of this reaction was thus of importance in relation to fatty acid synthesis in LC. Previous studies on the oxidative decarboxylation of pyruvate have revealed certain well-defined differences between the systems from different micro-organisms. LC is difficult to place taxonomically (Elsden *et al.* 1956), although it has recently been suggested that it should be placed in the genus *Peptostreptococcus* (Gutierrez, Davis, Lindahl & Warwick, 1959). It bears some biochemical resemblance to clostridia but is otherwise quite distinct from all the organisms previously used in studies of pyruvate breakdown.

The LC system was therefore also of interest from the standpoint of comparative biochemistry.

This paper describes the action of cell-free extracts of LC on pyruvate, and the general properties of the system. Portions of these findings have already appeared as brief reports (Peel, 1955, 1956, 1958*a*), in the latest of which it was suggested that pyruvyl-coenzyme A may be an intermediate in pyruvate breakdown. Further work has shown that the observations on which this interpretation was based were misleading because of the experimental conditions used, and this matter is dealt with fully in the present paper.

MATERIALS AND METHODS

Growth of the organism. The rumen organism LC, strain 1, of Elsden *et al.* (1956), National Collection of Industrial Bacteria no. 8927, was used throughout the present work. The maintenance and large-scale growth of the organism and the preparation of dried cells were as described by Walker (1958*a*), except that the harvested cells were washed in 10 ml. of 0·8 mM-sodium sulphide/g. of wet cell paste. The dried cells were pulverized and stored at $-20°$; preparations up to 4 years old still retain activity.

Cell-free extracts. Dried cells (2 g. portions) were extracted by suspending in 20 ml. of 0·01 M-potassium phosphate, pH 6·8, to which was added 0·8 mM-sodium sulphide to prevent oxidation by air. The suspension was incubated *in vacuo* at 37° for 1 hr., after which insoluble debris was removed by centrifuging at 12 000 *g* for 20 min. The resulting supernatant will be referred to as 'crude extract' and contained 20–30 mg. of protein/ml., as determined by the method of Bücher (1947). For dye-reduction experiments, crude extracts were treated further at 0° by adding 9 ml. of saturated ammonium sulphate/ml. of extract, centrifuging at 12 000 *g* for 20 min., discarding the supernatant and dissolving the precipitate in the phosphate–sulphide mixture used for extraction. This preparation will be referred to as 'ammonium sulphate-precipitated preparation'.

Both types of preparation were stored at 0° when in use, or at $-20°$ for longer periods; they were contained in tubes and discarded when the depth of liquid fell below 1 cm. Crude extracts were stable under these conditions and retained full activity after several months at $-20°$. The ammonium sulphate-precipitated preparations were less stable and lost up to 20% of their activity after 6 hr. at 0°. Duplicate samples stored at $-20°$ and tested after different periods up to several weeks had the same activity, but if a sample was tested, stored at $-20°$ and retested, up to 50% of the original activity was lost. It is concluded that some inactivation is associated with freezing and thawing. These preparations were therefore dispensed in 2–3 ml. portions and no portion was used on more than two occasions.

The preparations mentioned above were modified in some cases. For experiments on phosphate requirement, potassium phosphate was omitted from the medium used for extracting the dried cells and was replaced by 0·01 M-triethanolamine buffer, pH 6·8, in the medium used for dissolving the ammonium sulphate precipitate. For convenience, these preparations will be referred to as "phosphate-free'. Similarly, 'sulphide-free' denotes preparations obtained by omitting sulphide from the medium used for extraction and for dissolving the precipitate. When ammonium sulphate precipitation at pH 4·5 was required, 0·1 vol. of M-sodium acetate buffer, pH 4·5, was added to each volume of saturated ammonium sulphate. For precipitation at alkaline pH, 0·1 vol. of M-dipotassium hydrogen phosphate was added to 1 vol. of extract and this mixture and the saturated ammonium sulphate were adjusted to the required pH with N-sodium hydroxide before precipitation.

Dialyses were carried out at 2° and against 0·8 mM-sodium sulphide unless otherwise stated. Dialysis sacs of 0·75 in. diameter were placed in a chamber of 500 ml. capacity, which was rocked at 60 cyc./min. The chamber was fitted with an inlet and an outlet and, after it had initially been filled with the dilute sulphide solution, more was passed through from a reservoir at the rate of 1–2 l./hr.

Extract of boiled cells. Dried cells of LC were suspended in 10 ml. of water/g. of dried cells and heated at 100° for 10 min. Insoluble debris was removed by centrifuging for 20 min. at 12 000 *g* and the resulting extract stored at $-20°$ until required.

Coenzyme A, acetyl-coenzyme A and propionyl-coenzyme A. Coenzyme A (CoA), 75% pure, was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A. Acetyl- and propionyl-CoA were prepared from CoA by treating with the acid anhydrides and then purifying by paper chromatography, with acetate buffer in ethanol as the solvent (Stadtman, 1957). In each case a single thioester zone was obtained on the chromatogram. The adenine content of the eluate was taken as a measure of the amount of thioester present. The value from a corresponding blank strip of paper was less than 2% of that from the thioester zone.

Cofactor preparations containing acyl-coenzyme A. Early experiments on pyruvate oxidation by LC preparations in a dye-reduction system suggested that a new cofactor, present in extract of boiled cells, was required and the active material was purified from LC. Examination of the product showed that the principal active constituents were two thioesters of CoA, most probably the acetyl- and propionyl-derivatives. Cofactor concentrates from LC were subsequently used in several experiments reported here but their preparation will only be outlined because: (*a*) the purified cofactor could be replaced by synthetic acetyl- or propionyl-CoA, or, with a modified experimental system, by CoA itself; (*b*) there were indications that the batch of ion-exchange resin used at one stage of the purification was abnormal in its behaviour towards CoA and its derivatives, consequently the details of this part of the writer's procedure are not expected to be generally applicable.

The starting material was wet cell paste harvested from large-scale cultures of LC and was first extracted with trichloroacetic acid (final concn. 10%, w/v). The extract was treated essentially according to procedure B of Umbreit, Burris & Stauffer (1949) for phosphorylated intermediates, as far as the first barium precipitation. The precipitated barium salts were dried *in vacuo* and extracted with an excess of 0·1 N-sulphuric acid and the extract was then fractionated on a charcoal column as described by Stadtman & Kornberg (1953). The cofactor was eluted from the column with aq. 40% (v/v) acetone containing 0·017 N-ammonia. The later cofactor fractions were contaminated with flavins and the fractions were therefore combined into flavin-free and flavin-contaminated pools.

The flavin-contaminated pool was not purified further but was used in some experiments and will be referred to as 'crude cofactor'. The flavin-free pool was concentrated and then fractionated on a column of Dowex 1 chloride (Dow Chemical Co., Midland, Mich., U.S.A.; 200–400 mesh, 2% cross-linked). CoA derivatives were not eluted from the writer's batch of resin with formate–formic acid mixtures (cf. Stadtman & Kornberg, 1953), but a satisfactory fractionation was obtained by gradient elution with increasing concentrations of sodium chloride in 0·01 N-hydrochloric acid. Several nucleotide peaks appeared (detected by absorption at 260 mμ), and one of these, which was eluted with approx. 0·3 N-sodium chloride in 0·01 N-hydrochloric acid, contained almost all of the cofactor activity. This material was freeze-dried and freed of sodium chloride by applying it to a column of cellulose powder and eluting with 70% (v/v) ethanol, which removed the chloride before the cofactor. The product was freeze-dried and will be referred to as 'purified cofactor'. During purification, the cofactor was assayed by dye-reduction under the conditions of Fig. 6.

CoA assays on the purified cofactor (Kaplan & Lipmann, 1948; activity of pure CoA was taken as 316 units/μmole) gave a value of 1·1 mole of CoA/mole of adenine and determinations of pentose (Mejbaum, 1939) and total phosphate (Berenblum & Chain, 1938) gave a molar ratio for adenine: pentose: phosphate of 1:1·1:2·8. No free –SH could be detected qualitatively with nitroprusside (Stadtman, 1957) or quantitatively with *p*-mercuriphenylsulphonate (Boyer, 1954). The presence of a thio ester group was shown by the formation of hydroxamate on reacting with hydroxylamine, and by the decrease in extinction in the range 232–240 mμ and release of free –SH after alkaline hydrolysis (Stadtman, 1957). Quantitative determinations by these methods gave values of 0·5–0·7 equiv. of thioester/mole of adenine. Paper chromatography of the hydroxamates revealed two major spots in the positions of the acetate and propionate derivatives and a faint spot in the butyrate position. It was concluded that the purified cofactor was largely a mixture of thioesters of CoA and that the main components were probably acetyl- and propionyl-CoA.

When standard solutions of the purified cofactor were required, the thioester content was determined by the hydroxamate method.

Chemical reagents

All inorganic chemicals were of analytical-reagent grade unless otherwise stated. Triethanolamine buffer was prepared from potassium hydroxide and triethanolamine hydrochloride; the latter was made from triethanolamine (L. Light and Co. Ltd.) and recrystallized from water. Sodium arsenite solutions were prepared by dissolving resublimed arsenious oxide (E. Merck, Darmstadt, Germany) in a slight excess of sodium hydroxide and adjusting the pH to 7·5 with hydrochloric acid. A trace of material which did not dissolve was removed by filtration. Lithium acetyl phosphate was prepared by the method of Stadtman & Lipmann (1950) and was 79% pure on the basis of labile phosphate. A flavin concentrate, containing about equimolar amounts of flavin mononucleotide and flavin-adenine dinucleotide, was obtained from baker's yeast by following the separation previously described (Peel, 1958*b*) to the stage before electrophoresis.

Sodium pyruvate was prepared by the method of Robertson (1942) from pyruvic acid (L. Light and Co. Ltd.), which was first redistilled under reduced pressure. Solutions (0·1 M) were stored at −20° when not in use and in experiments where the amount of pyruvate was critical, freshly prepared solutions were used and standardized with yeast carboxylase as described by Umbreit *et al.* (1949).

2:6-Dichlorophenolindophenol was obtained from British Drug Houses Ltd.; 2:3:5-triphenyltetrazolium chloride, methylene blue ('pure, free from zinc chloride') and αα′-dipyridyl from E. Merck, Darmstadt; ethylenediaminetetra-acetic acid, iodoacetic acid, *p*-chloromercuribenzoic acid and 8-hydroxyquinoline were from L. Light and Co. Ltd.; carbon monoxide was from Imperial Chemical Industries; diphosphothiamine was from Roche Products Ltd.; sodium adenosine triphosphate was from Zellstoff-Fabrik Waldhof, Wiesbaden, Germany; diphosphopyridine nucleotide was from Pabst Laboratories, Milwaukee, Wis., U.S.A.; sodium triphosphopyridine nucleotide was from Sigma Chemical Co., St Louis, Mo., U.S.A. Corn-steep liquor and 70% (w/v) sodium lactate, used for large-scale cultures of LC, were obtained from Brown and Polson Ltd., Manchester, and British Drug Houses respectively.

The following were gifts: phenazine methosulphate (Dr V. Massey), DL-lipoic acid (Dr T. H. Jukes), lithium hydroxypyruvate (Mr D. H. Williamson), sodium glyoxylate (Dr H. J. Saz), sodium 2-oxoglutarate (Dr M. Kogut), flavin mononucleotide, 90% pure (Sigma Chemical Co.).

Measurements of pyruvate breakdown

Manometric measurements. Gas exchanges were measured in Warburg manometers by conventional techniques. The enzyme extracts were added last to the manometer cups, immediately before assembling and gassing.

Measurements of 2:6-dichlorophenolindophenol reduction. The reduction of this dye was measured in an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex) with a scale from 0 to 100 units. For the present purpose an optical cell was required in which anaerobic conditions could be established before the enzymic reaction was started. For various technical reasons, cells based on the Thunberg-tube design did not prove suitable and the apparatus eventually adopted was made as follows.

Pyrex test tubes, nominal dimensions 0·63 in. × 5 in., were selected which (*a*) were free from scratches, (*b*) fitted into the cell compartment of the colorimeter without sideways play, (*c*) gave a change of less than 0·5 scale unit on rotating the empty tube through 90° about a vertical axis, (*d*) gave scale readings within 1 unit for the different tubes when tested empty and with 6 ml. of a dye solution giving a scale reading of about 60 units. A side arm, similar to that of a Warburg manometer cup, was fused on to each tube about 4 in. from the bottom and the outer exit to the side arm could be sealed with a rubber valve of the Bunsen type. A convenient form of valve was made from a Subaseal cap (Turnover type, size 17; Subaseal Ltd., Barnsley) by piercing the centre with two slits in the form of a cross. The top of the main tube was closed with a rubber bung through which passed a glass tube for gassing the apparatus. This tube was fitted with a glass cock above the rubber bung and below the bung it extended to the bottom of the test tube. This lower portion of the gassing tube was of 3 mm. external diameter and its insertion was found to have negligible effect on the colorimeter reading with either

water or dye in the test tube. When this apparatus was inserted in the colorimeter, the normal cover of the cell compartment could not be closed and was replaced by an inverted cardboard box to exclude stray light.

Unless otherwise stated, all components of the reaction mixture except the enzyme were placed in the main tube of the apparatus. The gassing tube was then fitted and the apparatus flushed with oxygen-free nitrogen for 1 min. with the side arm open. The enzyme preparation was next placed in the side arm, the Bunsen valve was fitted and gassing continued for 1 min. longer. In a few cases where frothing occurred in the main tube during gassing it was overcome by placing a small smear of silicone high-vacuum grease (Dow Corning Corp., Midland, Mich., U.S.A.) on the gassing tube. After gassing, the apparatus was inserted in the colorimeter and readings were followed for at least 1 min. to measure any non-enzymic reduction (usually zero). The contents of the side arm were then tipped and further readings taken at appropriate intervals.

Experiments were carried out at room temperature, where this was 19–21°, otherwise the tubes were equilibrated in a water bath at 20° for at least 5 min. before placing in the colorimeter. Experimental runs used up to eight of these reaction tubes and gassing was timed so that approximately the same time interval elapsed in each case between gassing and the start of the enzymic reaction. Under these conditions no difference in activity was observed between duplicate tubes in the same experimental run.

The red OR1 filter was used and the instrument calibrated with standard solutions of the dye placed in one of the reaction tubes. Reaction volumes were always 6 ml. and contained about 0·3 μmole of the indophenol. The relationship between scale readings and the dye concentration was linear over the range used (1 scale unit $\equiv$ 5·2 μmoles of the indophenol in 6 ml.). During experiments it was found most convenient to set the zero of the instrument with a normal colorimeter cell containing 6 ml. of water. Because of this, the final reading at the end of the enzymic reduction (e.g. Fig. 2) represents a zero error and not unreduced dye.

For large-scale experiments with the indophenol, the following modifications were introduced. The reaction was carried out in a 50 ml. conical flask and nitrogen was bubbled through the reaction mixture throughout the experiment. The dye soln. (8·2 mM), from which oxygen had been removed by evacuation, was delivered from a 5 ml. burette, the tip of which dipped below the surface of the flask's contents. At the beginning, sufficient dye was run in to give a faint blue. The reaction was then started by adding the enzyme and, as the dye became reduced, more dye was added to maintain a constant blue colour. The reaction stopped when the pyruvate was exhausted, and the time taken was noted. A control without pyruvate was treated similarly for the same length of time, after which extra dye was added to bring the total amount added to the same as in the experiment with pyruvate.

Measurements with other hydrogen acceptors. When ferricyanide was used in place of the indophenol, the reaction was carried out as in the routine dye-reduction experiments unless otherwise stated. The colorimeter reading was small and was therefore used only as a rough guide to the progress of the reaction. When this had ceased, the ferricyanide remaining was determined spectroscopically by measuring the extinction at 420 mμ of a sample suitably diluted with water (ϵ $1{\cdot}01 \times 10^3$ l. mole^{-1} cm.$^{-1}$ for ferricyanide, 0 for ferrocyanide).

When other dyes were used as acceptors, the reaction tubes were calibrated in the same way as for the indophenol. With 2:3:5-triphenyltetrazolium chloride, where the oxidized form is colourless, a standard solution was converted into the red reduced form by adding sodium dithionite and then used for calibrating. This solution was slightly turbid, presumably owing to the low solubility of the reduced form, but no visible turbidity developed during the enzymic reduction of this acceptor. The recorded rate of tetrazolium reduction is therefore only approximate.

Analytical methods

Phosphate and acetyl phosphate. Inorganic orthophosphate was measured by the method of Fiske & Subbarow (1925). Certain labile phosphate compounds such as acetyl phosphate behave as inorganic phosphate in this assay. When a measure of labile phosphate was required, 'true' inorganic phosphate was determined by the method of Lowry & Lopez (1946) as modified by Peel & Loughman (1957) and labile phosphate was taken as the difference between the assays by this method and that of Fiske & Subbarow.

Acetyl phosphate was determined by the hydroxamate method as described by Lipmann & Tuttle (1945), except that the acetate-buffer reagent was replaced by water. When ferricyanide was present in incubation mixtures, this was first removed by adding 0·5 ml. of 0·25 M-zinc sulphate/ml. of sample, centrifuging off the precipitate and using a portion of the supernatant. A white precipitate which formed during the reaction with hydroxylamine did not interfere as it dissolved on adding the acid reagents.

Volatile fatty acids. These were separated by steam-distillation (Markham, 1942) after adding sulphuric acid to a final concentration of 1 N. Portions of the distillate were titrated with approx. 4 mN-sodium hydroxide in a carbon dioxide-free atmosphere with phenol red as indicator. Qualitative paper chromatography of the sodium salts was as described by Elsden & Lewis (1953). Quantitative chromatography was by the gas–liquid partition method of James & Martin (1952) with modifications according to Annison (1954); individual acids were identified by their retention volumes.

The indophenol and ferricyanide were found to interfere with volatile acid determinations and were therefore removed from incubation mixtures before steam-distillation, in the following manner. With the indophenol, the incubation mixture (about 30 ml.) was extracted twice with 40 ml. of ether. Sufficient 10 N-sodium hydroxide was then added to the aqueous layer to make it alkaline to phenolphthalein, after which it was evaporated to small bulk by boiling and then to 2–3 ml. in a stream of warm air from a hair drier. With ferricyanide, 5·5 ml. of reaction mixture was treated with zinc sulphate as for acetyl phosphate determinations, and a portion of the supernatant was made alkaline and evaporated as with the indophenol.

In large-scale experiments with the indophenol and ferricyanide, formate was determined on a sample of the neutralized distillate by the method of Leloir & Muñoz (1939) as modified by Barker (1957*b*).

Pyruvate. Incubation mixtures were deproteinized with trichloroacetic acid and pyruvate was determined by the

phenylhydrazone method as described by Friedemann (1957), the specific extraction procedure for monocarboxylic keto acids being used. When the indophenol was present in mixtures, this was largely adsorbed on the protein precipitate during deproteinizing and did not interfere in the determinations.

Glycollate. Incubation mixtures were treated with an equal volume of 4N-sulphuric acid, the precipitate was centrifuged off and a portion of the supernatant used for determination by the method of Dagley & Rodgers (1953). Hydroxypyruvate was inert in this assay.

Lipoic acid. Samples were pretreated and assayed according to Gunsalus & Razzell (1957).

Adenine. This was determined by measuring the extinction at 260 mμ in the presence of 0·01M-potassium phosphate, pH 6·8 (ϵ $15{\cdot}4 \times 10^3$ l. mole^{-1} cm.$^{-1}$).

Chromatography of hydroxamates. Hydroxamates, formed from acyl phosphates and CoA thioesters, were pretreated and separated on paper as described by Stadtman & Barker (1950). The solvent was either butanol saturated with water or butan-2-ol–90% (w/v) formic acid–water (75:13:12, by vol.).

Spectrophotometry. The Unicam (Cambridge) SP. 500 or the Zeiss (Oberkochen/Württ.) PMQ II instrument was used.

pH *measurements.* These were made with a glass electrode, with a pH meter, model 605 (W. G. Pye and Co. Ltd., Cambridge). Solutions containing saturated ammonium sulphate were diluted tenfold with glass-distilled water before measurement.

RESULTS

Metabolism of pyruvate

Action of crude extracts on pyruvate. When crude extracts of LC are incubated with pyruvate there is a rapid evolution of gas. The gas is a mixture of hydrogen and carbon dioxide, and by absorbing the latter the course of pyruvate breakdown can be followed manometrically by measuring hydrogen output (Fig. 1). With nitrogen as gas phase, hydrogen was evolved until the total output reached almost 1 μmole/μmole of pyruvate added; this was followed by a slight uptake of gas which, though small, was observed consistently (curve *A*, Fig. 1). In the absence of pyruvate there was no gas exchange (curve *B*).

Under hydrogen, the gas output from pyruvate was less than under nitrogen (curve *C*), but there was a considerable gas uptake in the blank without pyruvate (curve *D*). When corrected by algebraic subtraction of this blank, the total hydrogen output from pyruvate was the same as under nitrogen but the rate was a little lower. Nitrogen was consequently the more convenient gas phase and was the one normally used.

These gas exchanges are qualitatively similar to those observed when whole cells metabolize pyruvate (Elsden & Lewis, 1953), but with whole cells there is a large secondary uptake of hydrogen and the maximum gas output is no more than 0·5 μmole/μmole of pyruvate added. The gas exchange with whole cells is probably the net effect of a release of hydrogen in the oxidation of pyruvate and the utilization of hydrogen in the reductive formation of volatile fatty acids. With crude extracts this second reaction appears to be largely eliminated.

The activity of crude extracts varied considerably with the batch of dried cells, the best preparations giving initial hydrogen outputs of about 4 μmoles/hr./mg. of dried cells extracted. Extracts giving less than one-sixth of this rate were not used. For comparison, the initial output with whole cells is about 3 μmoles/hr./mg. dry wt. (calculated from data of Elsden & Lewis, 1953, assuming nitrogen content to be 10% of dry wt.).

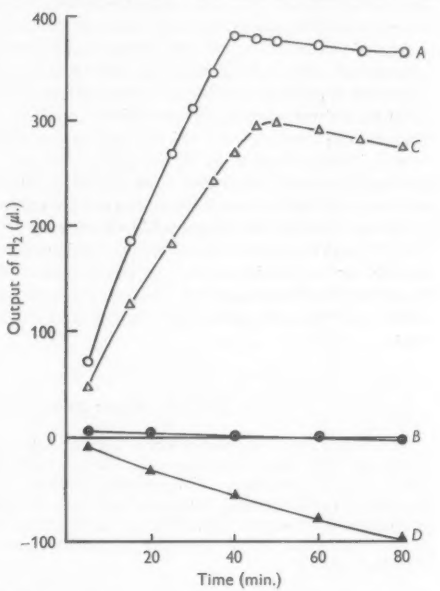

Fig. 1. Progress curves of pyruvate breakdown by crude extract under nitrogen and hydrogen. Manometers contained, in a total volume of 2 ml., 0·1M-potassium phosphate, pH 6·8, 0·4 ml. of crude extract and, where indicated, 18·2 μmoles of sodium pyruvate. The extract was originally placed in the side arm and tipped at zero time. The centre well contained 0·2 ml. of 10% KOH. Temp. 37°. Controls in which the crude extract had been heated at 100° for 5 min. are omitted for clarity; slight pressure changes occurred within the first 5 min. and corresponded to hydrogen outputs of 10 and 8 μl. under N_2 and H_2 respectively. Curve *A*, under N_2, with pyruvate; curve *B*, under N_2, no pyruvate; curve *C*, under H_2, with pyruvate; curve *D*, under H_2, no pyruvate.

Pyruvate oxidation with artificial hydrogen acceptors. The main hydrogen acceptor used was 2:6-dichlorophenolindophenol, the reduction of which gives a convenient and sensitive test for pyruvic dehydrogenase activity. Crude extracts catalyse a rapid reduction of this dye but often there is a large blank reduction in the absence of pyruvate, most of which occurs almost instantaneously on adding the enzyme. Ammonium sulphate-precipitated preparations always gave a low blank and were preferred for routine use. Such preparations require supplements of CoA or acyl-CoA and the course of the reaction is as shown in Fig. 2. All rates were measured over the linear phase and the activities of different preparations were in the range 1·0–3·5 μmoles of dye reduced/hr./mg. of dried cells extracted.

Tests with other hydrogen acceptors are summarized in Table 1. With methylene blue, flavin mononucleotide and the tetrazolium salt, the course of the reaction was similar to that with the indophenol, except that with the tetrazolium salt there was a preliminary lag of about 90 sec. These three acceptors were compared directly with the indophenol (Expt. 1, Table 1) and all reacted more slowly. The reduction of ferricyanide was tested spectrophotometrically; the rate of reduction decreased progressively as the reaction proceeded and in consequence the activity could be measured only roughly, but it is clear that ferricyanide is reduced rapidly by ammonium sulphate-precipitated preparations. The two pyridine nucleotides were also tested spectrophotometrically and found to be inert.

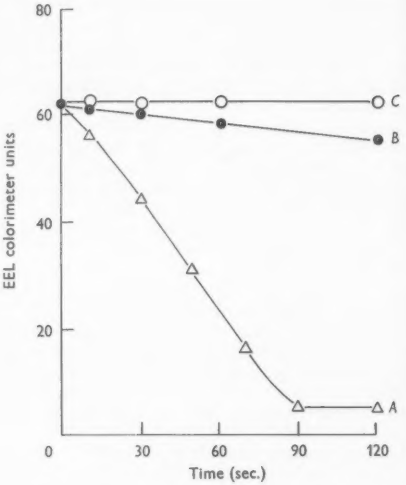

Fig. 2. Progress curve of the reduction of 2:6-dichlorophenolindophenol. The special reaction tubes described under Methods were used. They contained, in a total volume of 6 ml., 0·1 M-potassium phosphate, pH 6·8, 0·33 μmole of the indophenol, purified cofactor preparation containing about 0·1 μmole of acyl-CoA, 0·1 ml. of ammonium sulphate-precipitated preparation and, where indicated, 10 μmoles of sodium pyruvate. The enzyme was tipped from the side arm at zero time. Gas phase, N_2. Room temp. Curve *A*, with pyruvate; curve *B*, without pyruvate; curve *C*, enzyme heated at 100° for 10 min. The final reading with curve *A* represents zero error of the instrument and not unreduced dye (see Methods).

Table 1. *Pyruvate oxidation with various hydrogen acceptors*

Colorimetric tests were carried out as for Fig. 2, but with the hydrogen acceptor indicated. The same conditions were used in spectrophotometric tests except that half quantities were used, the cuvettes were not flushed with N_2 and, in tests with di- and tri-phosphopyridine nucleotide, 3 mM-cysteine was present; the reaction was started by adding the enzyme preparation to the cuvette. Numbers in parentheses after 'colorimetric' and 'spectrophotometric' indicate the filter or wavelength used respectively. The figures obtained for tetrazolium reduction are only approximate (see Methods). Rates were measured over the linear phase of the reaction, except with ferricyanide where the rate over the period 1–2 min. is given.

Expt. no.	Hydrogen acceptor	Concn. (μM)	Preparation	Method	Rate of reduction (μm-moles/min.) With pyruvate	Without pyruvate
1	2:6-Dichlorophenolindophenol	54	Crude extract	Colorimetric (OR 1)	227	10
1	Methylene blue	11	Crude extract	Colorimetric (OR 1)	100	2
1	2:3:5-Triphenyltetrazolium chloride	50	Crude extract	Colorimetric (OB 2)	33	0
1	Flavin mononucleotide	47	Crude extract	Colorimetric (621)	125	0
2	Phenazine methosulphate	4600	Ammonium sulphate-precipitated preparation	Colorimetric (OR 1)	No reduction	
3	Potassium ferricyanide	420	Ammonium sulphate-precipitated preparation	Spectrophotometric (420 mμ)	510	30
4	Diphosphopyridine nucleotide	45	Crude extract	Spectrophotometric (340 mμ)	No reduction	
4	Triphosphopyridine nucleotide	40	Crude extract	Spectrophotometric (340 mμ)	No reduction	

Effect of pH. This was investigated over the range covered by potassium phosphate buffers. The initial rate of hydrogen evolution by crude extracts showed a broad optimum centred around pH 6·8 (curve *A*, Fig. 3) and this pH was used in all subsequent experiments. For reduction of the indophenol the optimum pH was not established, but the change in activity between 6·8 and 8·0 was small (curve *B*, Fig. 3) and pH 6·8 was chosen for routine use to facilitate comparison of the results with those obtained by the hydrogen-evolution assay.

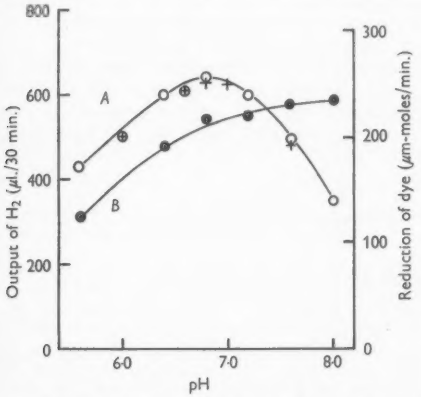

Fig. 3. Effect of pH on pyruvate breakdown. Curve *A*, H_2 evolution 0–30 min. Manometers contained 0·1 M-potassium phosphate buffer of the pH indicated, 40 μ-moles of sodium pyruvate and 0·4 ml. of crude extract. Gas phase, N_2. Other details were as described for Fig. 1. Data of two experiments are used (+ and ○). Curve *B*, dye-reduction. Reaction mixtures contained 0·1 M-potassium phosphate of the pH indicated, 10 μ-moles of sodium pyruvate. Other components and details were as described for Fig. 2. The extinction coefficient of the indophenol varies with pH, the value at pH 5·6 being half that at 6·8. The colorimeter was therefore calibrated empirically at each pH. Rates without pyruvate were measured at pH 5·6, 6·8 and 8·0. Hydrogen output was zero in each case. The rate of dye-reduction was 10 μm-moles/min. in each case; curve *B* is uncorrected.

Table 2. *Effect of concentration of crude extract on rate of hydrogen evolution*

Manometer cups contained 0·01 M-sodium pyruvate, 0·1 M-potassium phosphate, pH 6·8, and 0·2 ml. of crude extract; total final vol. 1, 2 or 4 ml. Gas phase, N_2. Other details were as for Fig. 1.

Concn. of crude extract (vol. of extract/total reaction vol.)	Hydrogen output (2–22 min.) (μl.)
0·05	39
0·1	84
0·2	107

Effect of concentration of enzyme preparations. When the effect of the amount of crude extract on the rate of hydrogen evolution was tested, with a constant reaction volume, the relationship was not linear, the rates with low amounts being disproportionately low. This dilution effect is shown more directly by the experiment of Table 2, in which the activity of a fixed amount of extract was found to vary widely with concentration. This suggested that cofactors were required and, on adding extract of boiled cells to the system, a stimulation was observed in some cases, e.g. in one experiment with 0·1 ml. of crude extract, the hydrogen output from pyruvate in the period 2–42 min. was increased from 45 to 100 μl. by 0·3 ml. of extract of boiled cells. With larger amounts of crude extract (0·4 ml.) the stimulation was smaller and was not obtained consistently; in some cases there was a slight inhibition. This may be caused by flavins in the extract of boiled cells, since LC has an unusually high flavin content (Peel, 1958*b*) and the hydrogenase of *Clostridium butyricum* is known to be inhibited by flavins (Peck & Gest, 1957). Because of these difficulties, most experiments on hydrogen evolution were done with a relatively large amount of extract and without the addition of extract of boiled cells.

With the ammonium sulphate-precipitated preparations, the rate of indophenol reduction was directly proportional to the amount of enzyme

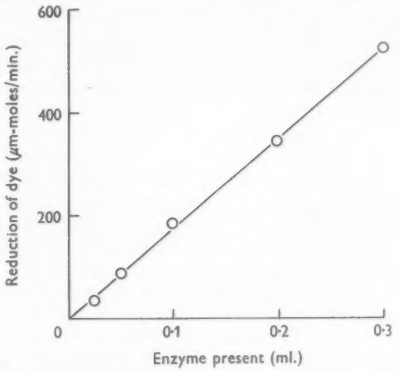

Fig. 4. Effect of enzyme concentration on the reduction of 2:6-dichlorophenolindophenol. Experimental details were as described for Fig. 2, except that the amount of ammonium sulphate-precipitated preparation was as indicated. Data are not corrected for rate in the absence of pyruvate; with 0·3 ml. of enzyme preparation this was 15 μm-moles/min.

present, over the convenient working range (Fig. 4).

Effect of phosphate. In preliminary experiments with crude extracts tested in triethanolamine buffer, a threefold stimulation of hydrogen output was obtained on adding phosphate, but the blank output without added phosphate was considerable and attempts to eliminate it by dialysis were only partially successful. Either little decrease was observed or the activity in the presence of phosphate was lost. The best effects were obtained with 'phosphate-free' preparations which had been dialysed for 3 hr., but the behaviour of these preparations was rather complex. CoA and Fe^{2+} ions were required to restore the activity to a level approaching that before dialysis but the gas output without phosphate was found to increase markedly with the CoA concentration (Table 3). This effect was catalytic in nature since 0·1 μmole of CoA increased this blank output by 4 μmoles. The dialysed preparation still contained some inorganic phosphate but this amounted to only 1·9 μmoles in the 0·4 ml. of extract employed, and the other reagents contained no inorganic phosphate. These findings suggest that there may be some breakdown of pyruvate by crude extracts which is independent of phosphate. At low levels of CoA, 0·1 M-phosphate increased the total hydrogen output about 17 times.

With the dye-reducing system, the effect of phosphate was more clear-cut and with some 'phosphate-free' ammonium sulphate-precipitated preparations an absolute requirement was obtained (Table 4). Potassium and sodium phosphate were equally effective, whereas potassium chloride was ineffective, confirming that the effect is due to phosphate rather than the cations (the same held true for the effect of phosphate on hydrogen evolution). Sodium arsenate at 0·01 M replaced phosphate, but higher concentrations were inhibitory.

Products of the reaction. In the manometric experiments, carbon dioxide was identified and estimated by the Warburg 'direct' method and the alkali-insoluble gas was presumed to be hydrogen since these are the only gases formed by growing cultures of LC (Elsden & Lewis, 1953). Crude extracts produce approximately equimolar amounts of each gas from pyruvate, together with one equivalent of volatile acid (Table 5). The volatile acids were examined by gas–liquid partition chromatography and, after allowing for the considerable blanks in the incubation without pyruvate, it was clear that the acid formed is acetic.

The volatile-acid determinations do not distinguish between the free acids and their labile acyl phosphates. The latter were therefore sought in two independent ways: by the hydroxamate

Table 3. *Effect of phosphate and coenzyme A on hydrogen production by dialysed extract*

Manometer cups contained, in a total vol. of 2 ml., 0·02 M-triethanolamine buffer, pH 6·8, 0·2 μmole of ferrous sulphate, 16·8 μmoles of sodium pyruvate, 0·4 ml. of 'phosphate-free' extract dialysed for 3 hr., and, where indicated, 0·1 M-potassium phosphate, pH 6·8, and CoA. Rates were measured over the first 20 min. Undialysed 'phosphate-free' extract tested with phosphate plus pyruvate gave 200 μl. of H_2/20 min. With the lower amounts of CoA, the rates could not be measured, as gas output ceased within a few minutes. Other details were as for Fig. 1.

	Hydrogen output			
	Rate (μl./20 min.)		Total (μl.)	
Coenzyme A added (μmole)	Without phosphate	With phosphate	Without phosphate	With phosphate
0	—	69	10	174
0·01	—	126	11	182
0·03	38	162	35	179
0·1	85	188	99	176

Table 4. *Effect of phosphate on reduction of* 2:6-*dichlorophenolindophenol*

Experimental details were as for Fig. 2, except for the following. The usual phosphate buffer was replaced by triethanolamine buffer (0·02 M in Expts. 1 and 3, 0·1 M in Expt. 2), together with the additions indicated. Phosphate and arsenate were added as mixtures of the monobasic and dibasic salts, pH 6·8. 'Phosphate-free' ammonium sulphate-precipitated preparations were used. A fresh extract prepared in the normal way from the batch of cells used for the extract of Expt. 3, and tested as in Fig. 2, reduced the dye at 206 μm-moles/min.

	Rate of dye-reduction (μm-moles/min.)				
	Expt. 1		Expt. 2	Expt. 3	
Additions	With pyruvate	Without pyruvate	With pyruvate	With pyruvate	Without pyruvate
None	21	5	0	21	5
0·1 M-Potassium phosphate	134	10	101	175	10
0·1 M-Sodium phosphate	150	10	—	—	—
0·1 M-Potassium chloride	21	5	—	—	—
0·01 M-Sodium arsenate	—	—	—	196	10

Table 5. *Products of pyruvate breakdown by crude extract*

Manometer cups contained sodium pyruvate as indicated. Gas phase, N_2. Carbon dioxide was measured by Warburg's direct method. For this purpose duplicate manometers without alkali in the centre well contained 0·5 ml. of 2 N-sulphuric acid in a second side arm, which was tipped at the end to release bound carbon dioxide. Initial acid 'tips' showed that the initial bound carbon dioxide was negligible. No residual pyruvate could be detected at the end, and no volatile acids other than formic acid, acetic acid and butyric acid were found. Other details were as for Fig. 1. All quantities are in μmoles.

	Pyruvate added	H_2 evolved	CO_2 evolved	Final volatile acid: Total	Formic acid	Acetic acid	Butyric acid
Expt. 1							
(*a*)	24·0	21·6	21·8	—	—	—	—
(*b*)	0	0	0·1	—	—	—	—
(*a* − *b*)	24·0	21·6	21·7	—	—	—	—
Expt. 2							
(*c*)	23·2	20·9	—	38·6	2·7	35·1	0·8
(*d*)	0	0·1	—	15·9	4·0	10·3	1·6
(*c* − *d*)	23·2	20·8	—	22·7	−1·3	24·8	−0·8

Table 6. *Acetyl phosphate as a product of pyruvate breakdown by crude extract*

Manometer cups contained 0·1 M-triethanolamine buffer, pH 6·8, 0·4 ml. of crude extract, 20 μmoles of potassium phosphate, pH 6·8, and sodium pyruvate as indicated. Gas phase, N_2. Other details were as for Fig. 1. Cup contents were assayed immediately after the gas output with pyruvate ceased (60 min.). Amounts are in μmoles.

	(*a*)	(*b*)	(*a* − *b*)
Pyruvate added initially	19·2	0	19·2
Acetyl phosphate by hydroxamate assay	16·4	2·0	14·4
Inorganic P + labile P	34·9	34·9	—
Inorganic P	20·4	35·7	—
Labile P (by difference)	14·5	−0·8	15·3

assay, which measures active acyl groups, and by measuring labile phosphate. About 0·75 μg.equiv. of hydroxylamine-reacting material was found for each μmole of pyruvate added, and paper chromatography of the hydroxamates showed that only the acetyl derivative was present (Table 6). An almost equivalent amount of labile phosphate was found and it is concluded that these assays represent acetyl phosphate. The yield was significantly less than the yield of either hydrogen or volatile acid. The experiment of Table 6 was carried out with only a little phosphate present, in order to give suitable conditions for the labile phosphate assay, but even when the usual 0·1 M-phosphate was used the yield of acetyl phosphate, measured by the hydroxylamine assay, was still low, never exceeding 80 % of the hydrogen output and being as low as 10 % with extracts from some batches of dried cells (such dried cells were not used further). It has been observed, moreover, that crude extracts destroy acetyl phosphate under conditions similar to those used in the manometric experiments. Thus in one experiment with 0·5 ml. of extract, 5 μmoles of acetyl phosphate disappeared in 30 min. It therefore seems highly probable that the breakdown of pyruvate by crude extracts, under the present conditions, proceeds mainly by the reaction:

$$H_3PO_4 + CH_3{\cdot}CO{\cdot}CO_2H \rightarrow H_2 + CO_2 + CH_3{\cdot}CO_2{\cdot}PO_3H_2 \quad (1)$$

followed by the hydrolytic breakdown of some of the acetyl phosphate.

In order to study the stoicheiometry of the reaction with the indophenol as hydrogen acceptor, it was desirable to scale up the reaction without increasing the volume unduly, but unfortunately the dye was found to be inhibitory at the higher concentration required. When the procedure was modified by titrating in the dye as the reaction proceeded (see Methods), the rate decreased progressively as more dye was added. Since the concentration of oxidized dye was small and roughly constant in this case, it would appear that the reduced form of the dye is also inhibitory. However, the titration method was more suitable for large-scale experiments as the dye-reduction could be followed more easily and the reaction was rapid in the initial stages. Conditions were chosen so that the final dye concentration required for complete oxidation of the pyruvate was about 0·4 mM (this concentration gives a rate equal to 40 % of the initial rate, when pyruvate is not limiting). Under these conditions, a close correspondence was found between pyruvate added, dye reduced and volatile acid formed (Expt. 1, Table 7). Examination of the volatile-acid fraction by paper chromatography revealed a single spot in the acetate position. Formate runs in the same position as acetate but its presence was excluded by separate determinations.

Table 7. *Products of pyruvate oxidation by ammonium sulphate-precipitated preparations*

In Expt. 1, the reaction mixture contained, in an initial volume of 28·5 ml., 3·0 ml. of M-potassium phosphate, pH 6·8, 0·5 mg. of crude cofactor preparation, pyruvate as indicated and 0·5 ml. of ammonium sulphate-precipitated preparation. The dye was titrated in and the reaction carried out as described under Methods. Reaction time, 26 min. Gas phase, N_2. Room temperature. In Expts. 2 and 3, a total reaction volume of 6 ml. contained 0·01 M-potassium phosphate, pH 6·8, pyruvate as indicated, 30 μmoles of potassium ferricyanide, 0·1 mg. of crude cofactor preparation and 0·2 ml. of ammonium sulphate-precipitated preparation. Reaction time: 45 min. in Expt. 2; 30 min. in Expt. 3. Room temperature. Values for ferricyanide reduced were obtained as the difference between the determined values for final ferricyanide and the amount originally added. Expt. 4 was done in manometers as for Fig. 1 except that the crude extract was replaced by 0·2 ml. of ammonium sulphate-precipitated preparation, and 0·1 mg. of crude cofactor preparation was added to the system. Incubated for 3 hr. at 25°. Amounts are in μmoles.

Expt. no.	Hydrogen acceptor		Pyruvate added	Acceptor reduced or H_2 evolved	Final volatile acid	Final acetyl phosphate
1	2:6-Dichlorophenol-indophenol	(*a*) Complete system	11·6	11·6	17·9	—
		(*b*) Control	0	0	6·7	—
		Difference (*a* − *b*)	11·6	11·6	11·2	—
2	Ferricyanide	(*c*) Complete system	9·6	17·6	15·2	—
		(*d*) Control	0	−0·9	4·8	—
		Difference (*c* − *d*)	9·6	18·5	10·4	—
3	Ferricyanide	(*e*) Complete system	9·6	19·5	—	1·6
		(*f*) Control	0	−1·1	—	0·9
		Difference (*e* − *f*)	9·6	20·6	—	0·7
4	Hydrogenase	(*g*) Complete system	9·6	7·2	—	6·8
		(*h*) Control	0	0·4	—	0·8
		Difference (*g* − *h*)	9·6	6·8	—	6·0

Experiments to decide whether the product of this reaction was acetyl phosphate proved inconclusive, as a satisfactory assay for acyl phosphate could not be devised under these conditions. The main difficulties were the low concentration of product relative to that of substances interfering with the hydroxamate assay, and the relatively high blank in controls without pyruvate. Attention was therefore turned to the products formed with ferricyanide as oxidant, since this behaves very similarly to the indophenol in other pyruvate-oxidation systems.

The stoicheiometry with ferricyanide was similar to that with the indophenol and the oxidation product was identified as acetate by the same means (Expt. 2, Table 7). Acyl phosphate was found in only small amounts of doubtful significance (Expt. 3) whereas acetyl phosphate added to duplicate incubation mixtures, either before or after the enzymic reaction, was recovered quantitatively. These experiments were done in 0·01 M-phosphate, as higher concentrations interfere with the hydroxamate assay, but semi-quantitative tests showed that no acyl phosphate was formed even with 0·1 M-phosphate.

In the absence of added oxidant, the ammonium sulphate-precipitated preparation used in these experiments catalysed a slow release of hydrogen from pyruvate. The total output was equivalent to only 70% of the pyruvate added, but it was accompanied by the appearance of an almost equivalent amount of acyl phosphate (Expt. 4, Table 7). It is thus evident that preparations capable of producing acetyl phosphate when releasing hydrogen from pyruvate, give the free acid when ferricyanide is used as oxidant.

Effect of inhibitors. The effect of inhibitors on pyruvate breakdown was examined first with the manometric assay, after which certain inhibitors were studied further with the dye-reduction test. Only –SH reagents and carbon monoxide showed potent effects; with the other agents tested the inhibitions were of doubtful significance because of the high concentrations required and the fact that sodium chloride inhibited at 0·1 M (Table 8).

The inhibitions obtained with iodoacetate, *p*-chloromercuribenzoate and arsenite indicate that –SH groups are involved in the reaction. The manometric system was appreciably less sensitive than the dye-reduction system, probably due to antagonism of the inhibitors by thiols present in the crude extract but absent from the ammonium sulphate-precipitated preparations. The sulphide present in the enzyme preparations does not antagonize the inhibitors, at least as far as the action of *p*-chloromercuribenzoate and arsenite on dye-reduction and of arsenite on hydrogen evolution are concerned, since essentially the same results were obtained with 'sulphide-free' preparations. The pyruvate-oxidizing system from LC is rather more sensitive to arsenite than the systems from *Proteus vulgaris*, *Lactobacillus delbrueckii* and *Clostridium butyricum*, which show little if any inhibition at concentrations of 10 mM or

Table 8. *Effect of inhibitors*

Rates of hydrogen evolution were measured over the period 0–20 min. with 20 μmoles of pyruvate, under the conditions of Fig. 1. The crude extract used in the experiments with carbon monoxide, ethylenediaminetetra-acetate and 8-hydroxyquinoline was highly active and 0·2 ml. was used so as to give convenient rates; otherwise 0·4 ml. was used. Dye-reduction was measured as in Fig. 2. Sufficient inhibitor was placed in the main body of the manometers or reaction tube to give the required final concentration, except with carbon monoxide, where this gas (or suitable dilutions in nitrogen) replaced the usual nitrogen used for flushing. The concentration of dissolved carbon monoxide was calculated from solubility tables, applying Henry's Law. Percentage inhibition was calculated as

$$\frac{(\text{rate without inhibitor} - \text{rate with inhibitor}) \times 100}{\text{rate without inhibitor}}$$

and no correction was made for rates in the absence of pyruvate. These were measured both without inhibitor and with the largest concentration of each inhibitor used, and never exceeded 7% of the rate with pyruvate but without inhibitor. The latter were from 190 to 417 μl./20 min. in the different manometric experiments and from 206 to 320 μmoles/min. in the different dye-reduction experiments.

Inhibitor	Concn. (mM)	Percentage inhibition: Hydrogen evolution	Percentage inhibition: Dye-reduction
Sodium iodoacetate	100	100	—
	5	73	—
Sodium *p*-chloro-mercuribenzoate	0·3	80	—
	0·1	2	98
	0·01	—	56
	0·001	—	7
Sodium arsenite	50	65	—
	10	—	82
	2·5	22	—
	1	—	56
	0·13	3	—
	0·1	—	14
Carbon monoxide	1	—	10
	0·8	97	—
	0·08	68	—
	0·008	9	—
Potassium cyanide	10	35	—
Sodium ethylene-diaminetetra-acetate	100	53	—
	10	—	0
	5	8	—
αα′-Dipyridyl	10	3	0
8-Hydroxyquinoline	1	—	0
	0·5	0	—
Sodium arsenate	100	52	See text
	5	17	—
Sodium fluoride	100	55	—
	10	15	—
Sodium chloride	100	52	41
	10	—	0

higher. It is nevertheless much less sensitive than the lipoic acid-dependent systems of *Streptococcus faecalis* and of animal tissues which are inhibited 50% at about 10 μM (Moyed & O'Kane, 1952*a*; Hager, Geller & Lipmann, 1954; Wolfe & O'Kane, 1953; Peters, Sinclair & Thompson, 1946).

Carbon monoxide, though strongly inhibiting hydrogen evolution, had almost no effect on dye-reduction, pointing to the hydrogenase system as the site of inhibition. The other metal-binding agents tested had little effect on hydrogen evolution. These results parallel the effects of these inhibitors on the hydrogenases of various organisms (Gest, 1952, 1954; Peck & Gest, 1957; San Pietro, 1955).

Except with carbon monoxide the test conditions were such that the inhibitors were not pre-incubated with the enzyme preparations and the results therefore give the instantaneous effect. There was no indication of any progressive increase in inhibition during the course of the reaction, except with arsenate in the dye-reduction test, where there was virtually no effect up to 0·01 M but a progressive inhibition at 0·05 M (Fig. 5). This effect will be discussed below.

Action on other substrates

No gas output was observed when crude extract was incubated with 10 μmoles of formate or 2-oxoglutarate under the conditions of Fig. 1, but without carbon dioxide absorber and with nitrogen

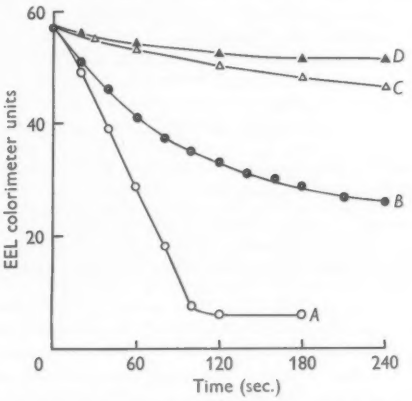

Fig. 5. Effect of arsenate on reduction of 2:6-dichlorophenolindophenol. Experimental details were as described for Fig. 2, except that the main tube also contained sodium arsenate, pH 6·8, final concn. 0·05 M, where indicated. Curve *A*, with pyruvate, no arsenate; curve *B*, with pyruvate, 0·05 M-arsenate; curve *C*, no pyruvate, no arsenate; curve *D*, no pyruvate, 0·05 M-arsenate.

as gas phase. With glyoxalate, a very slow gas output (10 μl. in 30 min.) was observed. Formate was also tested in the presence of 1 μmole of pyruvate, but the gas output was no greater than that expected from the pyruvate alone. With hydroxypyruvate, gas was evolved at a rate about half of that with pyruvate; hydrogen, carbon dioxide and glycollate were detected as products but the amounts were only roughly equivalent to each other and accounted for only about two-thirds of the substrate added (Table 9). This substrate also reduced the indophenol at about half the rate observed with pyruvate under the conditions of Fig. 2.

Lewis & Elsden (1955) reported the breakdown of 2-oxobutyrate by preparations of LC, and Walker (1958*b*) has examined its metabolism by crude extracts in more detail. The reaction is analogous to that with pyruvate; hydrogen, carbon dioxide and propionate are the products, with some of the propionate appearing as propionyl phosphate. In the dye-reduction test, 2-oxobutyrate reacts at about one-fifth of the rate with pyruvate.

Cofactor requirements

Coenzyme A. In early experiments on the reduction of the indophenol, it was found that the ammonium sulphate-precipitated preparations were inactive unless supplemented with extract of boiled cells. The extract could not be replaced by a mixed supplement of the known cofactors of pyruvate breakdown (Expt. 1, Table 10) and increasing the amounts of CoA or adenosine triphosphate in the supplement to 0·1 and 5·0 μmoles respectively was without effect. At this stage it was concluded that some new cofactor was involved and its purification from cells of LC was undertaken (see Methods). The purest preparation consisted mainly of two thioesters of CoA, believed to be the acetyl and propionyl derivatives, and could be replaced by acetyl- or propionyl-CoA prepared chemically from CoA (Expt. 2, Table 10). Treatment with alkali, which hydrolyses CoA thioesters to CoA and the free acid (Stadtman, 1957), destroyed the activity. When the purified cofactor, acetyl-CoA and propionyl-CoA were compared quantitatively on the basis of thioester content,

Table 9. *Metabolism of hydroxypyruvate by crude extracts*

Experimental details were in general as in Table 5, except that lithium hydroxypyruvate replaced sodium pyruvate. Gas phase, N_2. In both experiments the same batch of extract was used and gas exchanges ceased after 20 min. Expt. 1 was done in the absence of CO_2 absorber.

	Products (μmoles)		
	Expt. 1	Expt. 2	
	Glycollate	Hydrogen	Carbon dioxide
(*a*) No substrate	3·2	−0·1	1·9
(*b*) With hydroxypyruvate (10 μmoles)	9·4	7·6	7·4
(*b* − *a*) Difference	6·2	7·7	5·5

Table 10. *Cofactor requirement for reduction of 2:6-dichlorophenolindophenol*

Buffer, substrate, dye, enzyme and experimental procedure were as given in Fig. 2, with the supplements indicated placed in the main tube. The 'mixed supplement' contained the following (μm-moles): flavin concentrate 10, diphosphopyridine nucleotide 14, triphosphopyridine nucleotide 15, CoA 7, adenosine triphosphate 100, DL-lipoic acid 50, diphosphothiamine 20, $MgSO_4$ 10 000, $FeSO_4$ 30. The amounts of purified cofactor, acetyl-CoA, propionyl-CoA and hydrolysed supplements added each contained 20 μm-moles of total adenine. Samples of the supplements were hydrolysed by adding NaOH to give 0·1 N and then adding an equal volume of 0·1 N-HCl after 30 min. at room temp.; 0·6 ml. of the product was used.

	Rate of dye-reduction (μm-moles/min.)		
Supplements	With pyruvate	Without pyruvate	Supplement hydrolysed
Expt. 1			
None	20	—	—
Extract of boiled cells (0·3 ml.)	570	20	—
Mixed supplement	35	—	—
Expt. 2			
None	0	0	—
Purified cofactor	155	5	5
Acetyl-CoA	280	5	5
Propionyl-CoA	290	5	10

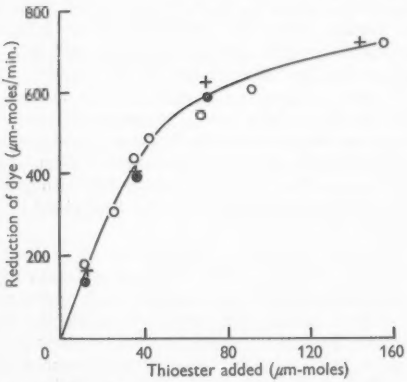

Fig. 6. Comparison of purified cofactor, acetyl-CoA and propionyl-CoA in the indophenol-reduction test. Experimental details were as described for Fig. 2, except that the indicated amounts of the different thioester preparations were used as cofactor. ○, Purified cofactor; ●, acetyl-CoA; +, propionyl-CoA. Values are uncorrected for rates without pyruvate; with the highest amount of purified cofactor used, this was 20 μm-moles/min.

Table 11. *Coenzyme A as cofactor for reduction of 2:6-dichlorophenolindophenol*

Experimental details were as described for Fig. 2, except that approx. 0·1 μmole of CoA was used as cofactor and the procedure was modified as follows. All components except CoA and the enzyme were placed in the main tube and flushed with N_2 for 1 min., after which the enzyme was placed in the side arm and the CoA was either placed in a second side arm or directly into the main compartment; gassing was continued for 1 min. longer. Pre-incubation time is the interval between adding CoA to the main compartment and the subsequent addition of the enzyme.

	Pre-incubation time (min.)	Rate of dye-reduction (μm-moles/min.) With pyruvate	Rate of dye-reduction (μm-moles/min.) Without pyruvate
CoA absent	—	5	—
CoA present	0	230	10
	2	95	—
	5	30	—
	10	25	5

they were equally active within experimental error (Fig. 6).

These experiments formed the basis of the previous report (Peel, 1958*a*) that acyl-CoA is needed in this system and cannot be replaced by free CoA. The writer is indebted to Dr W. Vishniac for pointing out that in the procedure adopted the CoA is pre-incubated with the indophenol and might be inactivated by non-enzymic oxidation, whereas acetyl-CoA would not react in this way.

Table 12. *Destruction of acyl-coenzyme A when pre-incubated with the indophenol and ammonium sulphate-precipitated preparation*

Components of the reaction mixture were as described in Fig. 2. The side-arm contents were as indicated and all other components were placed in the main tube. The enzyme was added to the apparatus last and the side-arm contents were tipped 20 min. later. In the experiment where the dye was placed in the side arm, the value for the colorimeter reading at zero time was obtained by backwards extrapolation of the progress curve; in all other cases the reading was taken immediately before tipping.

Side-arm contents	Rate of dye-reduction (μm-moles/min.)	Colorimeter reading at zero time
Enzyme	225	63
Pyruvate	5	49
Pyruvate + dye	195	62
Pyruvate + acyl-CoA	225	60
Pyruvate + enzyme	165	57

When the procedure was modified so that CoA could be added to the system at different times relative to the enzyme, it became clear that free CoA is active but only if added with, or soon after, the enzyme (Table 11). When pre-incubated with the other components, a slow reduction of about 0·05 μmole of the dye occurs and this supports the idea that the accompanying inactivation is due to the oxidation of CoA by the dye. The true situation thus appears to be that pyruvate breakdown in this system requires free reduced CoA, which may be replaced by acyl-CoA, but the disulphide form is inactive. It has not proved possible to counteract this inactivation of free CoA by the addition of a suitable reducing agent. The sulphide in the enzyme preparations is apparently ineffective and cysteine, glutathione, ferrous sulphate and ascorbic acid are all oxidized rapidly by the dye. In general it has been more convenient to retain the earlier procedure and supply the cofactor as acyl CoA.

Originally the enzyme was added from the side arm because much less, if any, activity was observed when the enzyme was placed in the main tube and pyruvate tipped from the side arm. This effect was now investigated further by placing various components in turn in the side arm with the pyruvate. With pyruvate alone in the side arm there was a complete loss of activity, whereas when the enzyme, the dye or the acyl-CoA was also put in the side arm only a part, or none, of the activity was lost (Table 12). Furthermore, the colorimeter readings immediately before tipping showed that with pyruvate alone in the side arm less oxidized dye remained. The conditions before tipping in this case are in fact the same as in curve *B* (Fig. 2), after tipping, where a slow blank reduction of the dye was observed. The inactivation thus involves

some interaction between the cofactor, the enzyme preparation and the dye, during which the dye is reduced and it seems most likely that some component of the enzyme preparation splits the cofactor to give free CoA, which is oxidized by the dye. The inactivation of the system is thus due to destruction of cofactor and there is no evidence that the apoenzymes are affected by dilution.

CoA markedly stimulates the rate of hydrogen output by dialysed preparations (Table 3), and Walker (1958*b*) has obtained evidence for an absolute requirement with preparations treated with Dowex 1.

Other coenzymes. The cofactor requirements for pyruvate breakdown in LC have been studied further with particular reference to diphosphothiamine, lipoic acid and metal ions. The possible need for diphosphothiamine in indophenol reduction has been investigated by treating crude extracts in the following ways: precipitating once with ammonium sulphate at pH 5·0, 8·0, 8·5 or 9·0; precipitating twice at pH 4·5; dialysing for 6 hr. against 0·01 M-dipotassium hydrogen phosphate containing 0·8 mM-sodium sulphide; dialysing for 3·5 hr. against 0·01 M-sodium acetate, pH 4·6, containing 0·8 mM-sodium sulphide. The resulting preparations were all active in the presence of acyl-CoA plus phosphate and no stimulation was observed on supplementing with diphosphothiamine plus extract of boiled cells. It would appear that if diphosphothiamine, or for that matter any other further cofactor, is required for indophenol reduction, then it is rather firmly bound to the apoenzyme.

The lipoic acid contents of preparations of LC have been determined and are given in Table 13, together with available data for pyruvate-oxidizing preparations from other organisms. Although the results relate to preparations of different degrees of purity and are therefore not strictly comparable, it is clear that the level of lipoic acid in LC is close to that in *C. butyricum* and well below that in *Escherichia coli*, *Streptococcus faecalis*, *Tetrahymena pyriformis* and animal tissues, where lipoic acid is involved in pyruvate oxidation. These results, together with the data on arsenite inhibition, indicate that lipoic acid is not involved in the LC system.

No LC preparation, including those mentioned above in connexion with diphosphothiamine, has so far required Mg^{2+} ions, and the lack of any substantial inhibition with fluoride supports the view that they are not involved. Wolfe & O'Kane (1953) likewise observed no magnesium requirement with *C. butyricum* but, in their system, Fe^{2+} ions were needed and could not be replaced by Fe^{3+} ions. It is extremely unlikely that there is any such requirement for indophenol reduction with the LC preparations because Fe^{2+} ions are unstable in this system. When 0·2 μmole of ferrous sulphate was added to the usual reaction mixture without enzyme, an equivalent amount of dye was reduced within 10 sec. The lack of inhibition with metal-binding agents also suggests that no free metal is involved in indophenol reduction.

On the other hand, some stimulation of hydrogen evolution by Fe^{2+} ions was observed with a dialysed preparation like that of Table 3. This preparation required CoA, and, on adding 10 mM-ferrous sulphate the hydrogen output in the period 0–45 min. was increased from 235 to 350 μl. Together with the inhibition by carbon monoxide, this suggests that iron is involved in the hydrogenase system.

Table 13. *Lipoic acid content of LC preparations*

1 unit is approximately equivalent to 20 $\mu\mu$moles of DL-lipoic acid.

Preparation from	Lipoic acid content (units/mg. of protein)	Reference
LC; crude extract: (*a*) aq. extract	0·08	—
(*b*) acid hydrolysate	0·15	—
LC; ammonium sulphate-precipitated preparation; aq. extract	0·035	—
Clostridium butyricum	0·05	Wolfe & O'Kane (1953)
Lactobacillus delbrueckii	< 1	Hager, Geller & Lipmann (1954)
Escherichia coli	200	
Tetrahymena pyriformis	10–35	Seaman (1954)
Animal pyruvic oxidase	212–1180	Schweet & Cheslock (1952)
	(units/mg. of dried cells)	
LC; aq. extract	0·007	—
Streptococcus faecalis	1*	Wolfe & O'Kane (1953)

* Level quoted by Wolfe & O'Kane as required for normal oxidation.

DISCUSSION

This investigation shows that the breakdown of pyruvate to acetate by crude extracts of the rumen organism LC is similar to that occurring in other strict anaerobes. Thus the formation of acetyl phosphate, carbon dioxide and hydrogen as products, together with the lack of formic hydrogenlyase activity, distinguish this system from those already studied in *L. delbrueckii* (Hager & Lipmann, 1955), *E. coli*, *Aerobacter* and *S. faecalis* (Ochoa, 1954; Gunsalus, 1954), *Proteus vulgaris* (Moyed & O'Kane, 1952*b*), *Mycobacterium tuberculosis* (Goldman, 1958*a*, *b*) and *Tetrahymena* (Seaman, 1953), and place it alongside those found in *Veillonella* (*Micrococcus lactilyticus*) (Whiteley & Ordal, 1957) and *Clostridium* (Koepsell & Johnson, 1942; Koepsell, Johnson & Meek, 1944; Nisman, 1954). The cofactor requirements of the clostridial system were studied extensively by Wolfe & O'Kane (1953), who used preparations from *C. butyricum* and found that diphosphothiamine, CoA and Fe^{2+} ions were needed. The indications were that magnesium and lipoic acid were not involved and the system did not reduce pyridine nucleotides; this particular combination of properties was unique among the organisms studied up to now. The LC system appears very similar to that of *C. butyricum* in all respects. The chief discrepancy is that no diphosphothiamine requirement has yet been shown with LC, but this cofactor is often firmly bound to the apoenzyme (Jansen, 1954) and the attempts to resolve the LC preparations cannot be regarded as exhaustive. It is also perhaps relevant that no enzyme system has yet been found which decarboxylates pyruvate without diphosphothiamine being involved.

The Fe^{2+} ion requirement in *C. butyricum* was demonstrated by using a tetrazolium derivative as hydrogen acceptor. Ferrous iron is not necessary for the exchange of $^{14}CO_2$ with the carboxyl of pyruvate (Wolfe & O'Kane, 1955) but appears to be required by the hydrogenase system (Peck & Gest, 1957). The results with LC are in keeping with the situation in *C. butyricum* if we assume that the ferrous-dependent component of the hydrogenase is necessary for tetrazolium reduction but not for indophenol reduction. In the lipoic acid-dependent systems, it has been suggested that the indophenol and ferricyanide can connect directly with the decarboxylating system, since, with these oxidants, lipoic acid and CoA are not obligatory and in their absence the decarboxylation product is free acetate and not acetyl phosphate (Gunsalus, 1954; Seaman, 1953). The action of the indophenol in the LC system is different in two respects. First, CoA is necessary, and secondly, the rate of dye-reduction is comparable with the rate of hydrogen production by both crude extracts and whole cells, whereas in the lipoic acid-dependent systems the indophenol and ferricyanide react only 0·1–0·001 times as rapidly as diphosphopyridine nucleotide, the natural acceptor (Gunsalus, 1954). Unfortunately the nature of the decarboxylation product when the indophenol is the oxidant in the LC system has not been established, though it is perhaps more likely that it is free acetate, since this is so with ferricyanide as oxidant and the behaviour of these two acceptors is similar in most systems. On the other hand, indophenol reduction does require phosphate.

Despite the uncertainties as to the action of the indophenol, it seems reasonable to assume at this stage that the enzymes and coenzymes required for its reduction are also part of the complete phosphoroclastic system. The use of the indophenol gives a convenient measure of pyruvate oxidation in LC and is superior to the manometric assay in that the stoicheiometry is more exact, hydrogenase is eliminated and the kinetics of the reaction are simpler. The present study illustrates the experimental difficulties arising with the indophenol, but of the oxidants so far tested only ferricyanide reacts as rapidly. This agent shares the disadvantage that it is rapidly oxidized by thiols and is less convenient technically. At present therefore 2:6-dichlorophenolindophenol seems the oxidant of choice.

The first experiments on cofactor requirements for indophenol reduction, in which acyl-CoA was active but not CoA itself, were interpreted as indicating that this reaction requires an energy-rich form of CoA and, on these grounds, it was suggested that pyruvyl-CoA, formed by a transphorase reaction, might be an intermediate in pyruvate breakdown (Peel, 1958*a*). The further experiments described here showing that CoA is active under suitable conditions demolish this line of evidence for such a pathway. However, the possibility that pyruvyl-CoA may be an intermediate remains open, and in this connexion it is of interest that Wiesendanger & Nisman (1954) obtained preparations from *C. saccharobutyricum* which required substrate amounts of adenosine triphosphate for pyruvate breakdown, suggesting perhaps that pyruvate is activated in some way. No such requirement has been observed with LC and attempts to test pyruvyl-CoA directly have proved inconclusive. Material obtained by acylating CoA with pyruvyl chloride was not active either as cofactor or substrate in the dye-reduction test, but the amount of product was too small to establish beyond doubt that it contained pyruvyl-CoA.

The fact that CoA is required for pyruvate breakdown and that it may be replaced by acetyl-CoA is in agreement with the view that acetyl-CoA is an intermediate in pyruvate breakdown in LC. The activity of propionyl-CoA is presumably due to its conversion into CoA or acetyl-CoA by the action of phosphotransacetylase or CoA transphorase (Stadtman, 1952, 1953). The formation of acetyl-CoA from pyruvate has not been directly demonstrated in LC but there is some indirect evidence for it. Although free CoA is inactivated within a few minutes by the indophenol, there is no sign of any decrease in the rate of dye-reduction during pyruvate breakdown, even when sub-optimum amounts of CoA are used as cofactor and the complete reduction of the dye takes 2 or 3 min. Apparently the CoA is protected against oxidation during the course of the reaction and a possible explanation is that the free CoA is rapidly acetylated in the presence of pyruvate. If this explanation is correct and if the enzyme preparations contain phosphotransacetylase, then the addition of arsenate would be expected to promote the arsenolysis of acetyl-CoA and so maintain a high ratio of free CoA to acyl-CoA. This should result in the oxidation of the free CoA by the indophenol and bring about a progressive inhibition of the reaction. This was in fact observed with arsenate (Fig. 5). Walker (1958*b*) has obtained evidence for the presence of a phosphotransacetylase in crude extracts of LC and the findings to date therefore support the theory that in LC the anaerobic oxidation of pyruvate yields acetyl-CoA, which in whole cells is available for the synthesis of higher volatile fatty acids but in crude extracts is converted into acetyl phosphate through the action of phosphotransacetylase.

The intermediate steps in the formation of acetyl-CoA from pyruvate in the strict anaerobes are at present obscure and it seems unlikely that rapid progress will be made until a purified enzyme system is available. Attempts to purify the *C. butyricum* system have so far been frustrated by its lability (Wolfe & O'Kane, 1953). The LC preparations used in the present study seem to be rather more stable and it is possible that this organism may offer a better opportunity for purification and further study.

SUMMARY

1. Under nitrogen at pH 6·8, crude extracts of dried cells of the rumen organism LC break down pyruvate to give approximately equivalent amounts of hydrogen, carbon dioxide and acetate.

2. The reaction is greatly accelerated by inorganic phosphate and most of the acetate appears as acetyl phosphate. There is, however, some hydrolysis of acetyl phosphate and a slow breakdown of pyruvate which appears to be independent of added phosphate but dependent on coenzyme A.

3. In the presence of pyruvate, ammonium sulphate-precipitated preparations rapidly reduce 2:6-dichlorophenolindophenol and ferricyanide. Crude extracts also reduce flavin mononucleotide, methylene blue and 2:3:5-triphenyltetrazolium, but less rapidly. Pyridine nucleotides are not reduced.

4. With ammonium sulphate-precipitated preparations the reduction of the indophenol is stoicheiometric. Phosphate (or arsenate) is required, but it is not clear whether the decarboxylation product is acetyl phosphate or free acetate. With ferricyanide as oxidant, free acetate is formed.

5. Reduction of the indophenol also requires coenzyme A. No other cofactor requirement has yet been found for this reaction, but there is evidence that Fe^{2+} ions are required for hydrogen production from pyruvate.

6. Coenzyme A may be replaced as cofactor by acetyl-coenzyme A or propionyl-coenzyme A. Free coenzyme A, but not acyl-coenzyme A, is oxidized non-enzymically by the indophenol and rapidly inactivated; consequently the activity of coenzyme A can be demonstrated only if precautions are taken to eliminate this effect.

7. Both hydrogen evolution and indophenol reduction are inhibited by –SH reagents. Carbon monoxide powerfully inhibits hydrogen evolution but not indophenol reduction; other metal-binding agents have little effect on either reaction.

8. Hydroxypyruvate and 2-oxobutyrate are metabolized in a manner similar to that of pyruvate but more slowly. No reaction was detected with formate or 2-oxoglutarate and only a very slow reaction with glyoxalate.

9. The pyruvate oxidation system in LC closely resembles that in other strict anaerobes, especially *Clostridium butyricum*.

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Substance SC (Ubichromenol): A Naturally-Occurring Cyclic Isomeride of Ubiquinone-50

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Lowe, Morton & Harrison (1953) reported that the unsaponifiable-lipid fraction from livers of vitamin A-deficient rats showed selective absorption in the ultraviolet region at 275 and 330 mμ. Two materials were concentrated by chromatography on alumina, a fraction with an absorption peak (in *cyclo*hexane) at 275 mμ and an inflexion near 330 mμ, and a second fraction showing peaks at 275 and 332 mμ with marked inflexions at 233 and 283 mμ. The latter has hitherto been designated 'substance SC' and it is now proposed to call it 'ubichromenol'. The former, known provisionally as 'substance SA' is now called 'ubiquinone' (Morton, Wilson, Lowe, & Leat, 1957; Morton, 1958; Morton *et al.* 1958) or coenzyme Q_{10} (Q_{275}) (Crane, Hatefi, Lester & Widmer, 1957; Wolf *et al.* 1958).

Substances SA and SC were found to be normal minor constituents of rat liver, kidney and intestine, and it was shown (Heaton, Lowe & Morton, 1955, 1957) that in this species vitamin A depletion brought about a pronounced rise in substances SA and SC in the liver and a slight rise in substance SC in the intestine. Substance SA was reported (Cunningham, Lowe, Mervyn, Morton & Vernon, 1955; Lowe, Morton & Vernon, 1957; Cunningham & Morton, 1959) in the liver and kidneys of many species. Substance SC was detected in the liver of many species and in the kidneys of most; it occurred in rather larger amounts in 'normal' human kidneys (Mervyn & Morton, 1958, 1959) but was not detected in nephritic kidneys.

Lowe (1953) and Heaton (1955) isolated, from the liver of vitamin A-deficient rats, small amounts of substance SC as a yellow oil showing absorption maxima at 233, 275 ($E^{1\%}_{1cm.}$ varying from 67·0 to 94·5), 283 and 332 mμ. Heaton found that substance SC could be acetylated, the acetate having an absorption peak at 317 mμ instead of 332 mμ. It contained no nitrogen, and SC acetate on analysis gave an empirical formula $C_{11}H_{17}O$. The Rast method indicated a molecular weight of 385 but this observation has been misleading (see below). The infrared-absorption spectrum of substance SC showed a hydroxyl band which disappeared on acetylation. The present paper describes the isolation and characterization of substance SC from human kidney.

MATERIALS AND METHODS

Materials. Human kidneys were made available by the help of Professor H. L. Sheehan, Department of Pathology, University of Liverpool, and were obtained from post-mortem examinations by Dr J. C. Brundret (Sefton General Hospital), Col. B. Blewitt (Newsham General Hospital), Dr C. A. St Hill (Royal Southern Hospital), Dr S. Whitwell (Broadgreen Hospital) and Dr H. E. Vickers (Walton Hospital), all in the Liverpool area. The kidneys were stored either at 0° or −30° until collection, which was generally within 2 days. After removal of the perirenal fat and membrane, and most of the pyramid fat, the kidneys were cut into pieces (about 2·5 cm.³).

Preparation of unsaponifiable extract. The kidney tissue was 'saponified' in 1 kg. portions. The tissue was added to a boiling mixture of 1 l. of 0·25% (w/v) pyrogallol in ethanol and 0·5 l. of aqueous 60% (w/v) KOH, and the mixture was refluxed for 45 min. After cooling, water (3 vol.) was added and the solution was extracted with 4 × 0·5 l. of light petroleum (b.p. 40–60°)–ether (4:1, v/v). The combined extracts were washed with water until free from alkali, dried over anhydrous Na_2SO_4 and filtered through a sintered-glass funnel; the filtrate was evaporated (finally under nitrogen) and the residue was weighed.

Crystallization of sterol. The unsaponifiable lipids were dissolved in the minimal volume of hot light petroleum (b.p. 40–60°) and the solution was left overnight at −30°. The sterol which had crystallized out was filtered off and washed with cooled light petroleum, the washings being added to the filtrate. The total filtrate was reduced in volume, cooled and a further crop of crystalline sterol was later removed.

Evaporation of solvents. Solvent was removed from each fraction by distillation on a boiling-water bath to a relatively small volume. This was transferred to a small conical flask and the remaining solvent was evaporated in a stream of nitrogen on a boiling-water bath, and the fraction carefully desiccated *in vacuo* and weighed.

Chromatography. Grade 0 alumina (P. Spence and Co., Widnes, Lancs.) was treated with N-HCl and washed with water until free from chloride. The alumina was dried by heating to 500° for ½ hr. and immediately before use was partially deactivated with water to Brockmann grade 3. The unsaponifiable material which remained after two crops of sterol crystals had been removed was chromatographed in 2 g. portions on alumina columns (100 g.). The solvents used to elute different fractions from the column were: light petroleum (b.p. 40–60°, dried over sodium wire and redistilled before use), diethyl ether (dried over sodium wire and redistilled from reduced iron) and methylal (redistilled). Usually five fractions were collected from the column:

(1) 1 l. of 3% ether in light petroleum (pre-ubiquinone fraction).

(2) 1 l. of 5% ether in light petroleum (ubiquinone fraction).

(3) 1 l. of 10% ether in light petroleum (SC fraction).

(4) 1·5 l. of 30% ether in light petroleum or 1 l. of ether (fraction containing sterol and vitamin A).

(5) 250 ml. of methylal (in some experiments only) (fraction containing sterol and vitamin A).

Substance SC was usually all in fraction (3) but some occasionally occurred in fractions (2) or (4), whereupon the particular fractions were rechromatographed on small columns.

Corresponding SC fractions from many runs were bulked and rechromatographed on alumina. The fractions rich in substance SC were then chromatographed on MgO (British Drug Houses Ltd.: 'for chromatographic analysis'), usually 0·5 g. of SC fraction on a 50 g. column. Solvents used to elute fractions from each column were: (1) 1 l. of 4% ether in light petroleum; (2) 1 l. of ether; (3) 250 ml. of methylal (SC fraction). If MgO from a freshly opened bottle is used a little substance SC may be eluted by ether but most of it is removed only by the more polar methylal.

Acetylation. Substance SC (100 mg.) was dissolved in pyridine (1 ml.) and the solution was cooled before the addition of excess (10 ml.) of acetic anhydride. The mixture was left overnight in the dark at room temperature, and was then poured into cold 5N-HCl (10 ml.). The mixture was extracted with light petroleum (3 × 10 ml.). The bulked extracts were washed with water until free from chloride and the solvent was removed.

Hydrogenation. Hydrogen uptake was determined in a microhydrogenation apparatus (J. W. Towers Ltd.). The solvent was acetic acid–ethanol–*cyclo*hexane (1:1:1, by vol.). Adams catalyst (PtO) was used and the hydrogen was at atmospheric pressure.

Spectrophotometry. Ultraviolet-absorption spectra were measured by a Unicam SP. 500 photoelectric spectrophoto-

meter. Unless otherwise stated, the figures reported refer to *cyclo*hexane solutions. Infrared-absorption spectra were measured with solvent-free films. The instrument was a Perkin–Elmer double-beam spectrometer, model 21 (Department of Organic Chemistry, University of Liverpool).

Nuclear magnetic resonance. The spectra were prepared by Dr L. H. Sutcliffe and Mr J. Feeney of the Department of Inorganic and Physical Chemistry, University of Liverpool. The materials were dissolved in $CHCl_3$, which was also used to provide the reference peak. The instrument was a Varian 4300-B nuclear magnetic resonance spectrometer run at 40 Mcyc./sec. All band measurements are positive shifts on the high-field side of the reference peak.

Analyses. These were carried out in the microanalysis laboratory of the Department of Organic Chemistry, University of Liverpool.

RESULTS

Yield of substance SC from human kidney. Human kidney (46·5 kg.) was saponified as described and yielded crude unsaponifiable material (140·75 g.). After two crystallizations, which yielded 92·9 g. of crude cholesterol, the residual unsaponifiable material, 41·2 g., was chromatographed in portions on alumina. The 3% of ether in light petroleum eluates contained 4·04 g. of material, $\lambda_{max.}$ 268 mμ. The ubiquinone-containing fractions eluted by 5% of ether in light petroleum yielded 5·12 g. of material, $\lambda_{max.}$ 272 mμ, $E^{1\%}_{1cm.}$ 100. The SC-containing fractions (eluted by 10% of ether in light petroleum) contained 9·32 g. of material with an inflexion at 233, $\lambda_{max.}$ 275 ($E^{1\%}_{1cm.}$ 11·1), inflexion 282 and $\lambda_{max.}$ 331 mμ. The subsequent fraction (15·87 g. eluted by ether) contained chiefly sterol with vitamin A as the principal absorbing entity, $\lambda_{max.}$ 327 mμ.

The SC-containing material (9·32 g.) was rechromatographed in portions on alumina, and yielded a ubiquinone-containing fraction (5·01 g.; $\lambda_{max.}$ 272 mμ, $E^{1\%}_{1cm.}$ 6·9) and a sterol fraction (1·87 g.). The SC fraction (1·91 g.) eluted by 10% of ether in light petroleum showed inflexion 233, $\lambda_{max.}$ 275 ($E^{1\%}_{1cm.}$ 29·3), inflexion 283 and $\lambda_{max.}$ 332 mμ. This last fraction was chromatographed on MgO. Light petroleum containing 4% of ether eluted 1·40 g. of material with negligible selective absorption. The material (0·441 g.) eluted by ether and methylal showed absorption peaks at 233, 275 ($E^{1\%}_{1cm.}$ 92·6), 283 and 332 mμ. Extraction of the adsorbent with hot methylal yielded 0·03 g. of material with no characteristic selective absorption.

The SC fraction (0·441 g.) was crystallized, first at −30° and then at 0°, from ethanol until the $E^{1\%}_{1cm.}$ values at 275 mμ of the crystals and mother liquors (separated by filtration at low temperature) were equal and constant. After seven crystallizations the yield of substance SC was 0·153 g. with $E^{1\%}_{1cm.}$ (275 mμ) 96·1 (see Fig. 1). These pale-yellow

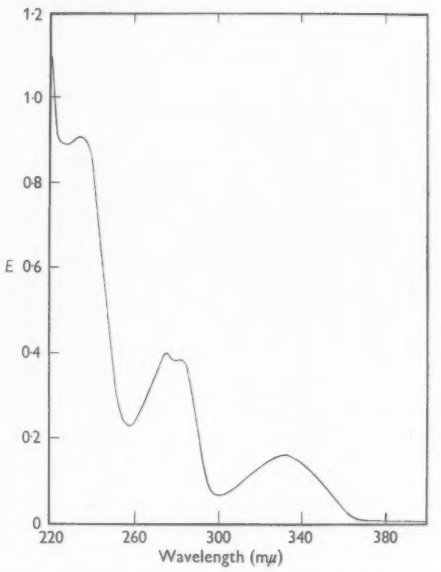


Fig. 1. Absorption curve of substance SC (ubichromenol), 0·00419% in *cyclo*hexane.

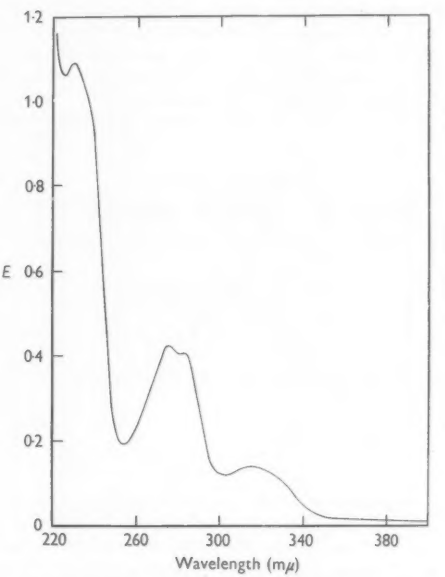


Fig. 2. Absorption curve of SC acetate (ubichromenyl acetate), 0·00459% in *cyclo*hexane.

crystals melted at about 18° but a precise melting point was not recorded. Further quantities of substance SC were obtained from the final mother liquors.

SC acetate. This was prepared as described and purified by chromatography on alumina. Substance SC (100 mg., nearly pure) gave acetate quantitatively and after crystallizations from ethanol yielded 60 mg. of white solid, m.p. about 18°, showing absorption peaks at 230, 275 ($E^{1\%}_{1\,cm.}$ 95·2), 285 and 314 mμ (see Fig. 2).

Infrared spectra. The infrared spectrum of substance SC is shown in Fig. 3*a* and clearly indicates the presence of an aromatic ring. An aromatic structure generally gives rise to bands near 3030 cm.$^{-1}$ (=C—H stretching vibration) and near 1600 and 1500 cm.$^{-1}$ (C=C vibrations), cf. Bellamy (1954, p. 54). Substance SC as a film shows bands at 3049, 3021 and 2994 cm.$^{-1}$, and also at 1605 and 1492 cm.$^{-1}$. Normally, aromatic structures show a weak band at 1580 cm.$^{-1}$, which is seen as a shoulder on the main 1600 cm.$^{-1}$ peak. In substance SC there is a distinct peak at 1575 cm.$^{-1}$ and this may indicate the presence of a double bond conjugated with the aromatic ring (cf. Bellamy, 1954, pp. 60–61). No clear indication of the degree of substitution of the aromatic ring of substance SC is given in the infrared spectrum.

Substance SC contains one or more hydroxyl groups, as is shown by the band at 3546 cm.$^{-1}$. Clearly there is no carbonyl group in substance SC, from the absence of the strong C=O band in the region 1650–1750 cm.$^{-1}$. The presence of one or more methoxyl groups is indicated by peaks at 1272 and 1098 cm.$^{-1}$. The bands at 1179, 1359 and 1389 cm.$^{-1}$ are in the region associated with skeletal vibration and CH deformation frequencies of *iso*propyl groups.

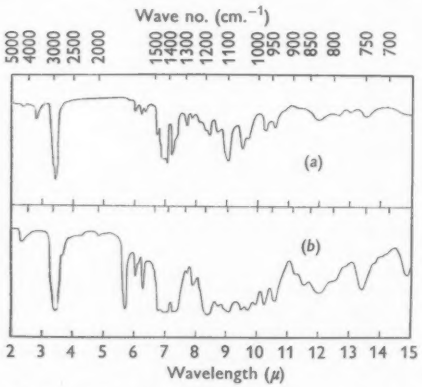


Fig. 3. Infrared-absorption curves: (*a*) substance SC (ubichromenol), film, minimum thickness; (*b*) SC acetate (ubichromenyl acetate), film, 0·075 mm.

The infrared spectrum of SC acetate is shown in Fig. 3*b*. In this spectrum the bands associated with hydroxyl groups are no longer present and the new bands at 1754 and 1190 cm.$^{-1}$ are indicative of a phenolic acetate.

Analyses. Substance SC: Found, C, 81·93; H, 10·67; O (direct), 8·05; OMe, 7·34%. SC acetate: Found, C, 80·81; H, 10·59; OMe, 7·05%. From the analyses it is apparent that substance SC contains four oxygen atoms, two being in methoxyl groups and one being in an acetylable hydroxyl group. The fourth oxygen atom cannot belong to a non-acetylable hydroxyl group (see infrared spectrum of SC acetate) nor to a carbonyl group (see infrared spectrum of substance SC). The C:H ratio of substance SC shows that the molecule must be considerably unsaturated. Its molecular weight, calculated from the analyses, is about 860 for four oxygen atoms, and therefore that of SC acetate would be 902. This makes substance SC very like ubiquinone-50 ($C_{59}H_{90}O_4$) which requires C, 82·08; H, 10·51; OMe, 7·2%. A monoacetate related to ubiquinone (I) would require C, 80·96; H, 10·24; OMe, 6·85%.

Hydrogenated substance SC and SC acetate. The hydrogen uptake of substance SC was 1·242 moles of hydrogen/100 g., which for a molecular weight of about 860 indicates 10·7 double bonds. For SC acetate the value was 1·307 moles/100 g., which for a molecular weight of 902 indicates 11·8 double bonds. These hydrogen uptakes for substances SC and SC acetate were reproduced, and there seems therefore no doubt that SC acetate takes up 1 mol.prop. of hydrogen more than does substance SC.

The ultraviolet-absorption spectra of hydrogenated substance SC (perhydro-SC) and hydrogenated SC acetate (perhydro-SC acetate) are given in Fig. 4, and their infrared spectra in Figs. 5*b* and 5*c* respectively. The maxima at 233, 275, 283 and 332 mμ of substance SC disappear on hydrogenation and in perhydro-SC are replaced by one maximum at 291 mμ, $E^{1\%}_{1\,cm.}$ 42·6. A double peak at 275 and 283 mμ of the intensity found in substance SC is consistent with it being an aromatic substance with one double bond conjugated with the ring (styrene type) (Laitinen, Miller & Parks, 1947). The disappearance of this double peak on hydrogenation would therefore be expected. The absorption maximum at 291 mμ of perhydro-SC and its intensity (molecular-extinction coefficient) are similar to those of aromatic substances with two oxygen functions in the *para* positions. Phenols, catechols and resorcinols all show peaks near 280 mμ (Burawoy & Chamberlain, 1952; Coggeshall & Lang, 1948; Hart & Haglund, 1950; Friedel &

Orchin, 1951), whereas quinols and tocols give rise to peaks near 290 mμ.

Supporting evidence for one phenolic hydroxyl group in substance SC is given by the effect of alkali on the ultraviolet spectrum. Perhydro-SC in ethanol shows $\lambda_{max.}$ 291 mμ, $E^{1\%}_{1cm.}$ 42 and on addition of sodium ethoxide there is a reversible shift to $\lambda_{max.}$ 308 mμ with a similar intensity (Fig. 6). This shift is consistent with salt formation by a phenol. The isosbestic points indicate a two-component system, i.e. one hydroxyl group. (We have observed that with two free hydroxyl groups in an aromatic ring there is a bathochromic shift in alkali but no isosbestic points are seen on the curves obtained with different amounts of alkali, e.g. 2-methylresorcinol in ethanol shows $\lambda_{max.}$ 274 and 280 mμ, $\epsilon_{max.}$ 1330, whereas in excess of alkali there is a single peak at 286 mμ, ε 2245.) With substance SC, alkali similarly causes reversible changes in the spectra, the peaks at 233 and 332 mμ moving to 246 and 358 mμ respectively (Fig. 7). We have already indicated that the double peak of substance SC at 275 and 283 mμ at the intensity observed is due to a styrene type of chromophore. It would seem that substance SC possesses an aromatic ring with a double bond conjugated to it, a free hydroxyl group in that ring and probably an oxygen function in the ring in the

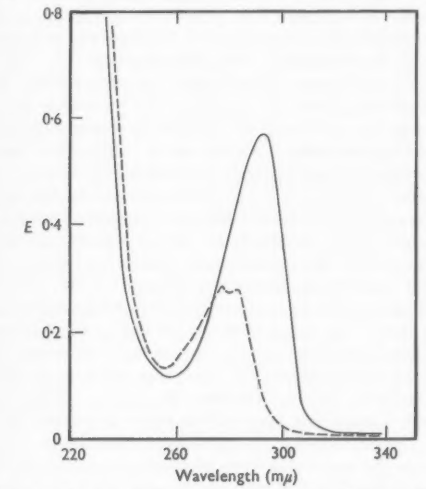


Fig. 4. Absorption curves: ——, perhydro-SC (perhydroubichromenol); - - - -, perhydro-SC acetate (perhydroubichromenyl acetate). Both 0·0133 % in *cyclo*hexane.

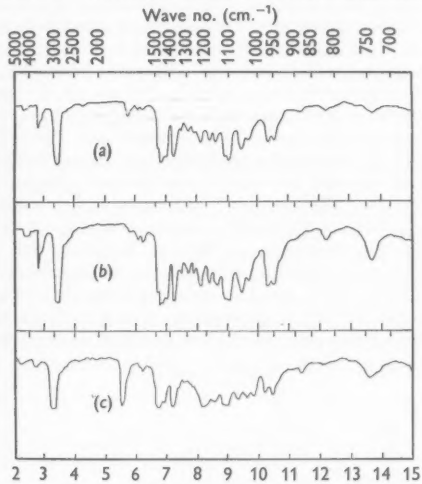


Fig. 5. Infrared-absorption curves: (*a*) cyclized derivative of ubiquinol, film, minimum thickness; (*b*) perhydro-SC (perhydroubichromenol), film, 0·025 mm.; (*c*) perhydro-SC acetate (perhydroubichromenyl acetate), film, minimum thickness.

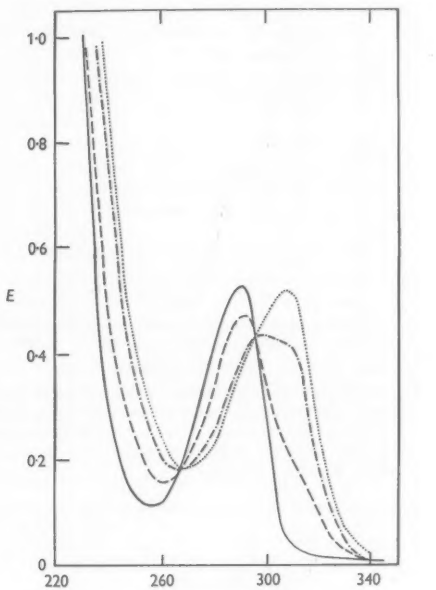


Fig. 6. Effect of sodium ethoxide on ultraviolet absorption of perhydro-SC (perhydroubichromenol). ——, Neutral solution, 0·0138 % in ethanol; - - -, 0·00217 M-sodium ethoxide; —·—·—, 0·0087 M-sodium ethoxide; · · ·, 0·0217 M-sodium ethoxide.

para position to the hydroxyl group. The ultraviolet-absorption spectrum of perhydro-SC acetate (two peaks 276 mμ, $E^{1\%}_{1cm.}$ 21·3; 282 mμ, $E^{1\%}_{1cm.}$ 20·9) resembles that of α-tocopheryl acetate and is consistent with substance SC having a similar type of structure.

In order to confirm the uptake of an additional mol.prop. of hydrogen by SC acetate, perhydro-SC was acetylated and further hydrogenation attempted. There was, however, no uptake of hydrogen. The material from this experiment showed $\lambda_{max.}$ 283 mμ, $E^{1\%}_{1cm.}$ 20·5 with a shoulder at 276 mμ, $E^{1\%}_{1cm.}$ 18·7. Thus acetylated perhydro-SC is similar to but not identical with perhydro-SC acetate.

The infrared spectrum of perhydro-SC acetate shows a weak hydroxyl band at 3546 cm.$^{-1}$ (Fig. 5c), which is absent from the otherwise similar infrared spectrum of the material recovered from the attempted hydrogenation of acetylated perhydro-SC. Since partial saponification in the hydrogenation procedure can be discounted, it must be assumed that a new hydroxyl group is formed on hydrogenation of SC acetate, and this must be associated with the uptake of an additional mol.prop. of hydrogen. It must further be concluded that the unidentified ether oxygen present in substance SC may be in a ring and that this ring splits on hydrogenation of SC acetate but not on hydrogenation of substance SC.

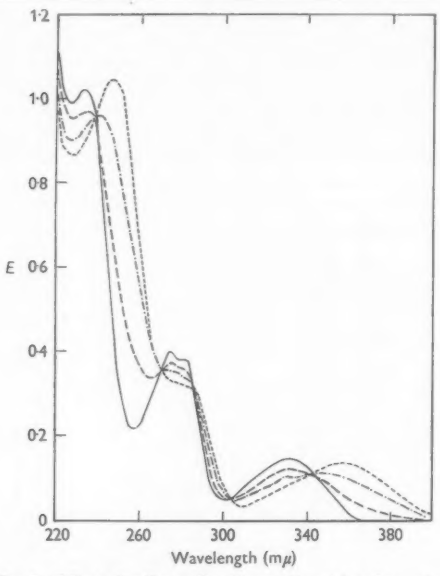
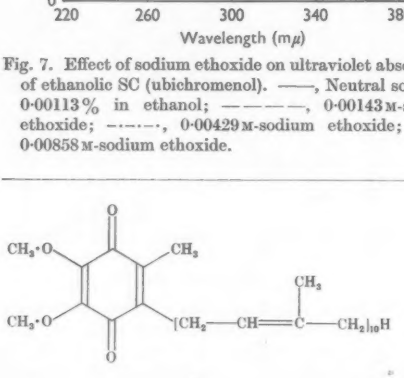
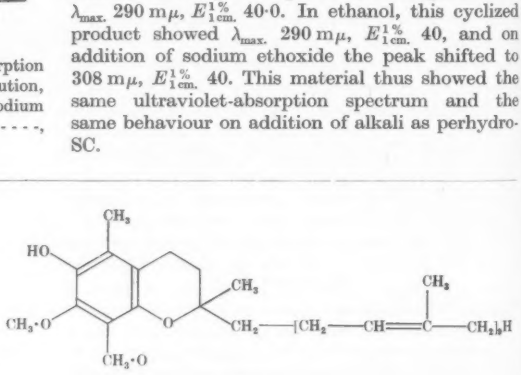


Fig. 7. Effect of sodium ethoxide on ultraviolet absorption of ethanolic SC (ubichromenol). ——, Neutral solution, 0·00113% in ethanol; — — — —, 0·00143 M-sodium ethoxide; –·–·–, 0·00429 M-sodium ethoxide; - - - -, 0·00858 M-sodium ethoxide.

A cyclized derivative of ubiquinone. So far substance SC appears to contain an aromatic ring with *para*-oxygen substitution, two methoxyl groups and a large unsaturated side chain, and these are also present in ubiquinol (Morton *et al.* 1957). Ubiquinol can be converted into a chroman-type of molecule by a cyclization involving the double bond of the first isoprene unit of the side chain and the hydroxyl group in the *ortho* position to this (Bouman, Slater, Rudney & Links, 1958). A structure of this type (II) is more in accord with the desiderata for the structure of substance SC, with the exception that there is no double bond conjugated to the ring, whereas there is evidence for this in substance SC. An attempted cyclization of ubiquinone by the method described for tocopheryl quinone by Bouman & Slater (1957) gave a very small yield. J. Green (personal communication) suggested that $SnCl_2$, concentrated HCl and acetic acid might prove a better reductive cyclizing reagent. In accordance with his procedure 0·2 g. of ubiquinone-50, 20 g. of $SnCl_2$, 200 ml. of HCl and 800 ml. of acetic acid were refluxed for 2–3 hr. on a boiling-water bath. This mixture was extracted with ether to yield a product which was crystallized from ethanol at $-30°$ after chromatography on alumina. The cyclized product, 114 mg., showed $\lambda_{max.}$ 290 mμ, $E^{1\%}_{1cm.}$ 40·0. In ethanol, this cyclized product showed $\lambda_{max.}$ 290 mμ, $E^{1\%}_{1cm.}$ 40, and on addition of sodium ethoxide the peak shifted to 308 mμ, $E^{1\%}_{1cm.}$ 40. This material thus showed the same ultraviolet-absorption spectrum and the same behaviour on addition of alkali as perhydro-SC.



(I)



(II)

The infrared-absorption spectrum of this cyclized product (Fig. 5*a*) is (apart from an unexplained peak at 1748 cm.$^{-1}$) virtually identical with that of perhydro-SC. Both of these spectra differ from that of substance SC in a few details, and these differences are largely in accord with the expected structures. Substance SC has a band at 1575 cm.$^{-1}$ not present in the cyclized product or in perhydro-SC, and, as already pointed out, this may be due to a conjugated double bond. Substance SC has a strong band at 1302 cm.$^{-1}$, which is much reduced in intensity in perhydro-SC and the cyclized product. This band is due to C–H stretching and deformation vibration of a *cis* double bond. If it is assumed that substance SC has an all-*trans* side chain, as in ubiquinone (Morton *et al.* 1958), the 1302 cm.$^{-1}$ frequency would be due to the double bond conjugated to the aromatic ring. The band at 1466 cm.$^{-1}$ in substance SC is less strong than that in the cyclized product and also in perhydro-SC. This band is in the region associated with the C—H deformation frequency of methylene groups.

There is no band at 1333 cm.$^{-1}$ in substance SC, whereas in the cyclized product there is a weak band and in perhydro-SC the band is stronger and quite distinct. This frequency is associated with the C—H deformation of a tri-substituted carbon atom (⩾C—H), which would arise in perhydro-SC on saturation of a polyisoprenoid side chain. The bands at 1416 and 833 cm.$^{-1}$ of substance SC are less intense in perhydro-SC. These frequencies in the region associated with C—H stretching and deformation vibrations of polyisoprenoid structures, $R_1R_2C{=}CHR_3$. However, in the cyclized product these bands are also weaker than in substance SC, and this is surprising since the cyclized product should have the same side chain as substance SC.

Oxidation of perhydro-SC. A ready confirmation of the supposed structure of substance SC would be the oxidation of perhydro-SC, to open the oxygen ring and give rise to perhydroubiquinone. Perhydro-SC (4 mg.) in 10 ml. of ethanolic solution of $FeCl_3$ (0·2 %, w/v) was kept overnight. The solution then was extracted with light petroleum and the extract showed $\lambda_{max.}$ 279 mμ, $E^{1\%}_{1cm.}$ approx. 160, $\lambda_{max.}$ 407 mμ, $E^{1\%}_{1cm.}$ 4·3 in ethanol. Authentic perhydroubiquinone shows $\lambda_{max.}$ 278 mμ, $E^{1\%}_{1cm.}$ 163, $\lambda_{max.}$ 407 mμ, $E^{1\%}_{1cm.}$ 4·3 (Morton *et al.* 1958). On addition of sodium borohydride to an ethanolic solution of the oxidation product, the spectrum showed $\lambda_{max.}$ 290 mμ, $E^{1\%}_{1cm.}$ approx. 50. Perhydroubiquinol has $\lambda_{max.}$ 290 mμ, $E^{1\%}_{1cm.}$ 49·5. Addition of further $FeCl_3$ reversed the changes. This is good evidence for the production of perhydroubiquinone by oxidation of perhydro-SC.

Nuclear magnetic resonance. The nuclear magnetic resonance spectra of ubiquinone-50 (human kidney), ubiquinone-30 (baker's yeast), substance SC and the cyclized product were examined. The conclusion drawn from the spectra by Dr Sutcliffe and Mr Feeney was that the nuclear magnetic resonance spectrum of substance SC fully supported the idea of ring closure.

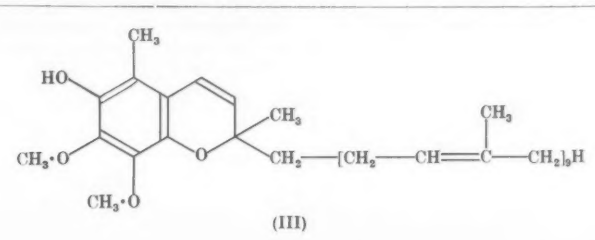
(1) The allyl hydrogens manifested in the spectra of ubiquinone-50 and ubiquinone-30, by peaks at 157 and 163 cyc./sec. and 161 and 167·5 cyc./sec. respectively, have disappeared in the spectrum of substance SC, as expected.

(2) In the spectra of the two ubiquinones the peak for the methoxyl group is single and in substance SC two methoxyl group peaks are shown: ubiquinone-50, 129 cyc./sec.; ubiquinone-30, 132 cyc./sec.; substance SC 132 and 133·8 cyc./sec. The two methoxyl groups in ubiquinone are equivalent whereas in substance SC they are non-equivalent.

(3) In substance SC, ring hydrogens are registered: 24·1 and 33·7 cyc./sec., and 61·8 and 72·0 cyc./sec., and the splitting of the two bands shows the two hydrogens in the ring to be on *ortho*-carbon atoms.

(4) In substance SC, a phenol hydrogen — 3·9 cyc./sec. is recorded. The high-field bands in substance SC, namely 208, 223·5 and 232 cyc./sec., are similar to those of ubiquinone-50 and -30 and it is reasonable to assume an isoprenoid side chain. Intensity measurements indicate that substance SC has nine isoprenoid units in the side chain (not counting the first isoprene unit which is involved in the ring).

All the evidence so far stated would agree with substance SC having structure (III).



(III)

The nuclear magnetic resonance spectrum of the cyclized product derived from ubiquinone showed that ring closure had taken place (the methoxyl groups were non-equivalent, allyl hydrogens had disappeared, phenol hydrogen had appeared) and also indicated that the heterocyclic ring hydrogens did not belong to an ethylenic linkage. Further, the spectrum showed that most of the unconjugated double bonds in the side chain had disappeared. The high field peaks, 190, 206, 226, 240 and 254 cyc./sec. are no longer similar to those obtained from the three previous compounds with the isoprenoid type of side chain. The peaks might indicate a linear saturated side chain but intensity measurements show that perhaps two double bonds remain.

The treatment of ubiquinone with $SnCl_2$ in a strongly acid medium appears to have effected the expected cyclization and in addition to have reduced some of the double bonds of the side chain, possibly with further cyclization.

DISCUSSION

The substance hitherto designated SC is somewhat widely distributed in animal tissues in quite small amounts. It first became accessible to study because its concentration in rat liver increased several-fold in advanced vitamin A-deficiency (Heaton *et al.* 1957). The evident structural relationship to ubiquinone which has now emerged might suggest that under these circumstances it was derived from ubiquinone by aberrant metabolism and that its accumulation reflects a metabolic block.

On the other hand, the best source of substance SC so far known is 'normal' human kidney, and its disappearance from the kidneys of patients who died from one form or another of nephritis (Mervyn & Morton, 1959) suggests that the metabolic abnormality lies in the absence of substance SC. This might result from failure to achieve synthesis or it might be the result of exhaustive hyperactivity in some process mediated by substance SC. It is too early to discuss how these two approaches to the biochemistry of substance SC are inter-related.

The structural investigation makes it appropriate to suggest the name *ubichromenol* for substance SC.

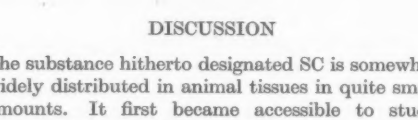
Kofler (1946) described a quinone from alfalfa and other plants which appeared to be a tetrasubstituted benzoquinone (m.p. 48–49°) of molecular weight about 800. It showed peaks at 254 and 262 mμ and a much weaker maximum near 320 mμ. Since the isolation of ubiquinone (coenzyme Q_{10}) Kofler's quinone has been studied afresh and it is now regarded as possessing the structure (IV), where $n = 9$. (Trenner *et al.* 1959; Kofler *et al.* 1959).

It thus differs from ubiquinone in lacking a methyl group at position 6 and in having methyl groups instead of methoxyl group at positions 2 and 3.

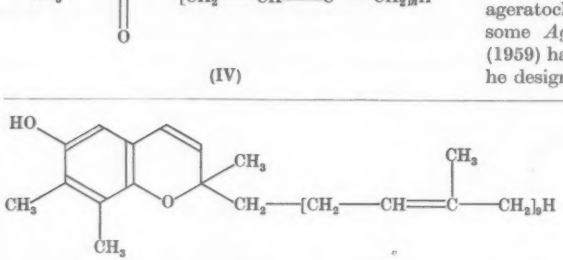
The occurrence in plants and animals of a polyisoprenoid side chain $C_{50}H_{81}$ has increased interest in the isolation by Rowland, Latimer & Giles (1956) of an allylic alcohol solanesol ($C_{45}H_{73}\cdot OH$) from tobacco leaves. The properties of this alcohol strongly suggest, however, that it is made up of nine isoprene residues joined together as in Kofler's quinone and ubiquinone 45 (Erickson *et al.* 1959; Kofler *et al.* 1959).

Rowland (1958) has since isolated from flue-cured tobacco a compound, solanachromene (V). The compound exhibits in the ultraviolet region absorption peaks at 233, 267 (inflexion 275) and 332 mμ ($\log \epsilon$, 4·37, 3·76, 3·74 and 3·63 respectively). This absorption spectrum is consistent with that of ubichromenol, where the double peak in the middle ultraviolet region is displaced towards longer wavelengths and increased a little in intensity.

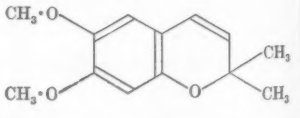
The chromene ring has also been found in ageratochromene (VI) from the essential oils of some *Ageratum* species (Alertsen, 1955). Crane (1959) has discovered that Kofler's quinone, which he designates Q_{254}, is concentrated in chloroplasts,



(IV)



(V)



(VI)

whereas mitochondria from the same leaves contain coenzyme Q_{10} (ubiquinone-50). The association of the former with photosynthetic electron transport and of the latter with mitochondrial electron transport provides some guide for future work on solanachromene and ubichromenol.

Interesting possibilities arise from this work, namely that tocopherol analogues with polyisoprenoid side chains may well be found in nature with the chromene structure. The naturally occurring vitamins K with polyisoprenoid side chains may well be accompanied by or be in metabolic relationship with cyclized derivatives.

SUMMARY

1. A normal minor unsaponifiable lipid, hitherto designated substance SC, and present in kidney and other tissues of many species, has been isolated from normal human kidney, its concentration in this tissue being about 10 mg./kg.

2. Substance SC is a yellow crystalline compound, m.p. about 18°. It is isomeric with ubiquinone, $C_{59}H_{90}O_4$, but is not itself a quinone.

3. Substance SC contains a chromene ring system and a large polyisoprenoid side chain. Perhydro-SC can be oxidized by ferric chloride to perhydroubiquinone. Substance SC is therefore a cyclic isomeride of ubiquinone, the cyclization involving the first isoprenoid unit of the side chain and the adjacent oxygen function.

4. The name ubichromenol is suggested for substance SC.

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Renal Clearances of Amino Acids in Normal Adults and in Patients with Aminoaciduria

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The development of new methods of amino acid analysis during the past 15 years has resulted in a very large increase in our knowledge of the way in which these important constituents are handled by plant and animal tissues and organisms. Of these new methods, ion-exchange chromatography has many advantages when quantitative measurements are required, as it is capable of much greater precision than paper-chromatographic techniques and is more specific than microbiological methods. In addition, microbiological methods can only give results for the number of compounds for which assays are done; no information is obtained about the presence of, or changes in, unknown or unexpected amino acids, and this is a serious disadvantage, especially when pathological material is being examined. The main disadvantage of the ion-exchange method at present is that it is time-consuming and laborious. The advent of a fully automatic self-recording modification of the ion-exchange-column method (Spackman, Stein & Moore, 1958) should enable this objection to be overcome. The many discoveries which have been made with these new methods in the field of human amino acid excretion have been reviewed by Dent (1952*a*), Harris (1955) and Harrison & Harrison (1957), and will not be discussed in detail here.

The majority of measurements which have been made so far in man with the ion-exchange method have been based on 24 hr. urine collections (e.g. Stein, 1953) or isolated plasma samples (Stein & Moore, 1954). Evered (1956), however, has reported approximate renal amino acid clearances in normal humans and in patients with certain metabolic diseases which were calculated by comparing 24 hr. urine excretions with the concentrations of amino acids in a plasma sample taken on a different day. Sereni, McNamara, Shibuya, Kretchmer & Barnett (1955) calculated fasting renal clearances of individual amino acids in normal premature babies, infants, children and adults with estimates of plasma and urine amino acid concentrations obtained from paper chromatograms. Doolan, Harper, Hutchin & Shreeve (1955) and Doolan, Harper, Hutchin & Alpen (1956), using microbiological methods, have measured renal clearances of amino acids in normal human adults in acute experiments both in the fasting state and during an amino acid infusion. Arrow & Westall (1958), using techniques identical to those employed in the present study, have reported from this Department renal clearances in normal adults and in patients with cystinuria as well as in the heterozygous carriers of the disease.

The studies reported here were undertaken to confirm or otherwise previous theories of the mechanism of amino acid excretion (Dent, 1954) and to elucidate further the mode of amino acid excretion in certain metabolic diseases.

EXPERIMENTAL

Subjects studied. Physical data are given in Table 1, and further clinical details are in the Appendix.

Collection of samples. Subjects were fasted overnight and rested in bed for at least an hour before collections were commenced, until the end of the urine collection. Water was given to drink (about 250 ml. every 30 min.) from an hour before collections commenced and throughout the experimental period to maintain a reasonable urine flow and so minimize bladder-emptying errors. An accurately timed 3 hr. sample of spontaneously voided urine was collected; catheterization was not done. Blood samples of about equal volume were taken from an arm vein with a dry syringe at $\frac{1}{2}$, $1\frac{1}{2}$ and $2\frac{1}{2}$ hr. after the start of the urine collection. Each was transferred to a heparinized tube, centrifuged immediately and plasma removed. Plasma and urine samples were stored at $-10°$ until required for analysis. The collection was arranged to coincide with a time when blood was required for routine biochemical tests whenever possible, especially in the children. In the children also the number of venepunctures was reduced to two, at 1 and 2 hr.

Preparation of samples. Urine was applied to the analytical column without preliminary treatment. At the end of the collection period equal volumes of the two or three plasma specimens from the subject were mixed to give a single sample, which was then prepared by the method of Stein & Moore (1954) by deproteinizing with picric acid and removing the picric acid from the protein-free filtrate with a small column of Dowex 2 anion-exchange resin in chloride form.

Amino acid analysis. The method of Moore & Stein (1954*a*) was employed, a 0·9 cm. × 150 cm. column of Dowex 50 X 4 cation-exchange resin in the sodium form being used and gradient elution with sodium citrate buffers in the range pH 3·1–5·1. Fractions (2 ml.) were collected

Table 1. *Details of subjects studied*

Subject	Sex	Age (years)	Height (cm.)	Weight (kg.)	Surface area ($m.^2$)	Diagnosis
D.A.W.	M.	27	168	59·6	1·66	Normal
D.C.C.	M.	28	171	63·2	1·73	Normal
H.G.R.	F.	21	173	61·3	1·72	Normal
C.H.	F.	29	164	73·9	1·81	Normal
V.C.	F.	24	139	35·8	1·18	Osteomalacia with increased glycine excretion (Type I of Dent, 1952*b*)
E.G.	M.	37	167	56·8	1·54	
S.K.	M.	7	116	24·8	0·90	Renal-tubular rickets with glycosuria (Type II of Dent, 1952*b*)
L.R.	F.	46	147	47·0	1·35	Nutritional osteomalacia
M.P.	F.	40	147	44·3	1·35	Adult hypophosphatasia
E.Hu.	F.	46	148	60·3	1·58	Chronic renal failure with secondary hyperparathyroidism
M.Y.	F.	56	161	51·0	1·53	Adult Fanconi syndrome
J.J.	M.	7	91	13·2	0·61	Cystinosis
G.J.	F.	9	110	20·3	0·80	Cystinosis
E.Ha.	M.	17	153	46·0	1·39	Hartnup disease
K.R.	M.	7	118	21·0	0·88	Argininosuccinic aciduria

with a drop-counting collector and analysed by the photometric ninhydrin method of Moore & Stein (1954*b*).

Estimation of glomerular filtration rate. Creatinine concentrations of some samples of plasma and urine were estimated during the amino acid analysis by collecting 1 ml. fractions in the region in which creatinine emerges from the column and analysing alternate fractions with the ninhydrin reagent referred to above, and with alkaline picrate (Jaffe, 1886), which is much more sensitive to creatinine. It was possible to measure plasma-creatinine concentrations in this way in those subjects with renal damage with elevated plasma-creatinine concentrations, but the method was not sensitive enough to make accurate plasma measurements in those subjects with normal glomerular function. For these subjects, who had normal renal function as judged clinically and from biochemical evidence such as blood urea and urea clearances, an estimate of glomerular filtration rate was obtained from body-surface area, with reference values 127 ml./min./1·73 $m.^2$ and 118 ml./min./1·73 $m.^2$ respectively for males and females (Smith, 1951). For the subjects for whom a plasma-creatinine value was obtained, the endogenous creatinine clearance was calculated. Although it is fully appreciated that neither of these methods gives an accurate value for glomerular filtration rate, it was not felt that the more complicated procedure for inulin clearances was justified, as the values were required for approximate calculation and comparative purposes only. Moreover the presence of inulin in the samples might have interfered with the amino acid analysis. With these estimates, calculations were made of the load of amino acids filtered at the glomerulus and the fractions of this load excreted and reabsorbed. Owing to their approximate nature, they are not presented in detail but are discussed in the text. Body-surface area was estimated from height and weight with the chart given by Sendroy & Cecchini (1954).

RESULTS

Plasma concentration, urinary excretion and renal clearance of individual amino acids are given in Tables 2–4. Glutamine emerges as a mixed peak with asparagine and is also unstable on the column, recoveries being in the range 25–50% (Moore & Stein, 1954*a*), but a large deviation from values obtained in normal subjects presumably reflects a real difference in the sample. Tryptophan is also unstable on the column, but in none of the samples examined here has a definite peak been seen in this position, so that no values are reported for this amino acid. Throughout the analyses of urine samples, and to a much smaller extent in the plasma samples, small unidentified peaks were frequently encountered, but no attempt has been made to characterize them here. These probably represent some of the many unidentified amino acid-like compounds which are known to occur in human urine (Westall, 1955).

Normal subjects

The values reported here are in good agreement with previous work by ion-exchange chromatography (Evered, 1956) and microbiological assay (Doolan *et al.* 1955). Evered (1956) has compared the results obtained by ion-exchange analysis with those obtained by other methods and this will not be discussed further here. Approximate calculations of the filtered loads of the individual amino acids indicate that 99% or more of most of the amino acids are normally reabsorbed by the renal tubules, 95–98% of glycine is reabsorbed and 90–95% of histidine. The reabsorption of taurine appears to vary considerably, ranging between 88 and 99% in the four subjects studied here. Moderate amounts of 1- and 3-methylhistidines and of β-amino*iso*butyric acid may be excreted while the plasma concentrations of these compounds is very low. Although it is impossible to measure accurately such low plasma values by the present methods, the results suggest that relatively small proportions of these amino acids are reabsorbed by the renal tubules, leading to a high rate of renal clearance.

Table 2. *Plasma amino acid concentrations (mg./100 ml.)*

See text for additional values and details. In this and subsequent tables amino acid abbreviations are those of Brand & Edsall (1947) with the addition of: Tau, taurine; Gln, glutamine + asparagine; α-AB, α-amino-*n*-butyric acid; β-AB, β-amino*iso*butyric acid; 1 mH, 1-methylhistidine; 3 mH, 3-methylhistidine. A dash (—) indicates that a result was not obtained for technical reasons; a nought (0) indicates that a peak was not detected or was too small to be identified or measured with certainty; figures in parentheses indicate that the result is approximate.

	Normal adults				Severe Type I osteomalacia		Type II rickets	Nutritional osteomalacia
Subject ...	D.A.W.	D.C.C.	H.G.R.	C.H.	V.C.	E.G.	S.K.	L.R.
Tau	0·88	0·79	0·96	1·3	0·73	0·81	—	1·3
Asp	0·09	0	0	0	0	0	—	0
Thr	2·0	2·0	1·5	1·3	1·5	1·8	0·81	1·7
Ser	1·4	1·2	1·3	1·3	1·1	1·3	0·70	1·3
Gln	2·6	3·2	4·0	4·5	3·6	3·1	0·42	3·9
Pro	1·7	2·0	2·6	1·9	3·2	5·5	2·1	2·4
Glu	1·3	1·3	0·56	1·1	0·61	0·94	1·3	1·9
Gly	1·8	2·2	2·0	2·1	2·0	2·6	1·9	1·8
Ala	2·3	3·3	3·6	2·4	2·5	4·9	3·4	3·5
α-AB	0·29	0·22	0·18	0·24	0·24	0·27	0	0
Val	3·3	2·7	2·3	2·4	2·2	2·5	2·0	1·6
Cys	1·5	0·63	0·88	0·20	0·76	1·3	1·3	0·80
Met	—	—	0·27	—	0·42	0·30	0·22	0·21
iLeu	0·87	0·89	0·70	0·61	0·53	0·81	0·61	0·67
Leu	1·7	1·4	1·3	1·2	1·3	1·5	1·2	0·98
β-AB	0	0	—	0	(0·015)	0	(0·03)	0
Tyr	0·88	0·87	0·99	0·64	0·69	0·93	0·67	0·77
Phe	0·91	0·84	0·90	0·80	0·80	0·96	0·77	0·75
Orn	1·2	0·84	0·99	0·63	0·46	1·1	0·46	0·67
Lys	2·4	2·4	2·1	2·3	2·0	2·3	1·5	2·1
His	1·3	1·5	1·2	1·2	1·2	1·5	1·2	1·3
1 mH	0·29	(0·09)	(0·05)	—	0	0·47	(0·1)	0·35
3 mH	0	(0·06)	(0·05)	—	0	0	(0·1)	0·14
Arg	1·0	2·4	1·4	1·3	0·65	2·3	1·2	1·2

	Adult hypophosphatasia*	Hyperparathyroidism*	Adult Fanconi syndrome	Child Fanconi syndrome with cystinosis		Hartnup disease	Argininosuccinic aciduria*
Subject ...	M.P.	E.Hu.	M.Y.	J.J.	G.J.	E.Ha.	K.R.
Tau	1·1	0·97	0·77	0·78	2·3	0·72	—
Asp	0	0·17	—	0·26	0·12	0·12	—
Thr	0·58	0·92	1·4	0·91	1·2	0·84	1·7
Ser	1·6	0·92	0·98	0·84	0·87	1·1	1·5
Gln	2·5	1·8	4·5	2·5	2·0	1·7	4·1
Pro	4·9	3·1	5·3	3·1	3·2	3·7	2·7
Glu	0·77	1·0	2·3	1·0	1·1	1·2	1·1
Gly	1·5	1·3	2·8	1·5	1·4	1·4	3·6
Ala	3·9	3·6	3·7	3·0	3·0	2·5	2·8
α-AB	0·25	0	0·19	0·17	0·18	—	0·19
Val	3·0	1·6	2·0	1·7	1·9	2·2	1·7
Cys	1·5	2·6	1·1	1·4	1·4	1·0	0
Met	0·17	—	—	0·21	—	0·17	0·39
iLeu	0·84	—	—	0·62	—	0·82	0·66
Leu	1·5	1·0	1·2	1·0	1·3	1·6	1·3
β-AB	0	0·12	0	0·15	0·26	0	—
Tyr	1·1	—	1·0	0·78	0·60	0·66	2·2
Phe	0·98	—	1·0	0·90	0·96	0·97	1·0
Orn	0·82	0·90	0·70	0·47	0·48	0·79	0·44
Lys	2·1	1·5	1·9	1·6	1·6	3·0	1·9
His	1·3	1·0	1·3	0·74	0·93	1·5	1·4
1 mH	0·39	1·0	0·46	0·34	1·2	0	—
3 mH	(0·08)	0·40	0	0·18	0·29	0	—
Arg	2·0	0·98	1·7	1·1	1·0	1·7	0·61

* See text for additional values and details.

Table 3. *Amino acid excretions* (*μg./min./1·73 m.²*)

See Table 2 for explanation of abbreviations.

	Normal adults				Severe Type I osteomalacia		Type II rickets	Nutritional osteomalacia
Subject ...	D.A.W.	D.C.C.	H.G.R.	C.H.	V.C.	E.G.	S.K.	L.R.
Tau	90	51	130	22	140	120	27	115
Asp	2·2	—	0	0	8·1	—	6·3	0
Thr	17	19	22	10	18	19	15	62
Ser	27	38	37	24	10	49	29	96
Gln	18	58	24	30	6·8	25	16	86
Pro	0	0	0	0	0	0	0	1·5
Glu	4·6	4·2	3·4	8·0	11	11	6·0	13
Gly	49	91	116	89	340	260	180	300
Ala	10	30	26	6·9	21	42	28	64
α-AB	0	2·9	1·9	0	3·0	—	3·5	3·3
Val	4·4	8·9	1·4	0·9	2·6	3·0	3·2	7·8
Cys	10	4·5	10	5·7	6·2	—	10	0
Met	—	3·7	3·1	3·4	4·3	7·4	7·9	7·1
iLeu	1·8	8·0	3·9	6·1	1·1	6·5	3·1	5·3
Leu	4·0	3·5	12	3·4	5·5	4·3	1·9	3·4
β-AB	14	12	12	9·1	34	20	8·4	—
Tyr	15	15	10	8·6	8·6	14	14	24
Phe	6·5	8·1	12	11	12	10	18	9·4
Orn	0	0	0	0	0	0	0	0
Lys	45	5·2	28	11	15	7·3	12	56
His	120	115	79	58	120	120	150	255
1mH	51	32	41	26	36	38	59	180
3mH	—	44	27	18	35	45	17	32
Arg	8·0	7·5	4·3	2·2	5	13	0	3·7

	Adult hypophosphatasia*	Hyperparathyroidism*	Adult Fanconi syndrome	Child Fanconi syndrome with cystinosis		Hartnup disease	Arginino-succinic aciduria*
Subject ...	M.P.	E.Hu.	M.Y.	J.J.	G.J.	E.Ha.	K.R.
Tau	150	23	94	14	146	98	64
Asp	14	4·5	—	7·8	6·7	20	—
Thr	2·7	43	310	49	113	470	7·0
Ser	14	45	260	53	104	920	16
Gln	44	120	1030	91	210	1300	13
Pro	4·3	120	800	110	180	0	0
Glu	40	6·6	67	15	26	65	8·3
Gly	70	86	590	130	180	220	58
Ala	25	120	820	145	240	580	6·6
α-AB	3·3	2·0	30	3·2	7·2	19	—
Val	8·0	32	370	62	100	350	—
Cys	8·4	78	300	69	98	32	—
Met	9·7	3·2	24	2·8	—	38	—
iLeu	3·8	11	120	19	43	210	—
Leu	3·9	23	200	29	58	240	—
β-AB	15	7·4	—	28	47	—	—
Tyr	22	27	190	32	54	320	25
Phe	13	30	180	31	56	200	6·5
Orn	1·5	19	150	15	22	0	—
Lys	3·6	44	420	56	110	200	24
His	100	54	300	61	96	950	8·3
1mH	27	61	110	16	110	—	23
3mH	35	29	—	18	16	—	4·8
Arg	17	15	290	—	50	—	—

* See text for additional values and details.

Table 4. *Renal clearances of amino acids (ml./min./1·73 m.²)*

See Table 2 for explanation of abbreviations.

	Normal adults				Severe Type I osteomalacia		Type II rickets	Nutritional osteomalacia
Subject ...	D.A.W.	D.C.C.	H.G.R.	C.H.	V.C.	E.G.	S.K.	L.R.
GFR	127	127	118	118	118	127	127	118
Tau	10	6·4	14	1·7	19	15	—	9·2
Asp	2·4	—	—	—	—	—	—	—
Thr	0·9	1·0	1·5	0·8	1·2	1·1	1·8	3·6
Ser	2·0	3·1	3·0	1·9	1·0	3·7	4·2	7·4
Gln	0·7	1·8	0·6	0·7	0·2	0·8	3·8	2·2
Pro	0	0	0	0	0	0	0	0·06
Glu	0·4	0·3	0·6	0·7	1·8	1·2	0·5	0·7
Gly	2·7	4·1	5·8	4·3	17	10	9·8	16
Ala	0·4	0·9	0·7	0·3	0·8	0·8	0·8	1·8
α-AB	0	1·3	1·1	0	1·1	—	—	—
Val	0·1	0·3	0·06	0·04	0·1	0·1	0·2	0·5
Cys	0·7	0·7	1·2	2·9	0·8	—	0·7	0
Met	—	—	1·1	—	1·0	2·5	3·5	3·4
iLeu	0·2	0·9	0·6	1·0	0·2	0·8	0·5	0·8
Leu	0·2	0·2	0·9	0·3	0·4	0·3	0·2	0·3
β-AB	—	—	—	—	—	—	—	—
Tyr	1·7	1·7	1·0	1·4	1·2	1·5	2·1	3·2
Phe	0·7	1·0	1·3	1·4	1·5	1·1	2·4	1·3
Orn	0	0	0	0	0	—	0	0
Lys	1·9	0·2	1·3	0·5	0·7	0·3	0·8	2·7
His	9·7	7·9	6·6	4·7	9·9	7·9	13	19
1mH	17	—	—	—	—	8·1	—	50
3mH	—	—	—	—	—	—	—	23
Arg	0·8	0·3	0·3	0·2	0·7	0·6	0	0·3

	Adult hypophosphatasia*	Hyperparathyroidism*	Adult Fanconi syndrome	Child Fanconi syndrome with cystinosis		Hartnup disease	Arginino-succinic aciduria*
Subject ...	M.P.	E.Hu.	M.Y.	J.J.	G.J.	E.Ha.	K.R.
GFR	118	7·3	29	23	15	127	127
Tau	14	2·4	12	1·8	6·5	13	—
Asp	—	2·7	—	2·9	5·5	17	—
Thr	0·5	4·7	22	5·4	9·6	56	0·4
Ser	0·9	4·9	27	6·4	12	85	1·1
Gln	1·8	6·7	23	3·6	11	74	0·3
Pro	0·09	3·9	15	3·5	5·6	0	0
Glu	4·0	0·8	2·9	1·5	2·4	5·4	0·7
Gly	4·6	6·8	21	8·5	12	16	1·6
Ala	0·7	3·3	22	4·8	8·0	23	0·2
α-AB	1·3	—	16	2·0	4·1	—	—
Val	0·3	2·0	19	3·7	5·4	16	—
Cys	0·6	3·0	27	4·8	7·2	3·0	—
Met	6·0	—	—	1·4	—	22	—
iLeu	0·5	—	—	3·0	—	26	—
Leu	0·3	2·2	16	2·9	4·5	15	—
β-AB	—	6·4	—	18	18	—	—
Tyr	2·0	—	19	4·1	9·0	49	1·1
Phe	1·3	—	18	3·4	5·8	21	0·6
Orn	0·2	2·2	22	3·1	4·6	0	—
Lys	0·2	2·9	22	3·5	7·4	6·8	1·3
His	7·8	5·4	22	8·3	10	66	0·6
1mH	7·0	6·1	23	4·4	8·9	—	—
3mH	—	7·1	—	9·8	5·5	—	—
Arg	0·9	1·6	17	—	4·9	—	—

* See text for additional values and details.

Severe osteomalacia with increased glycine excretion

The urine results fully confirm previous findings of increased glycine excretion in these cases (Dent & Harris, 1956; Evered, 1956), and the plasma concentrations and clearance values suggest that this increased excretion is of renal origin. The plasma glycine of patient V.C. was within normal limits and the renal clearance was considerably elevated above the normal range. Patient E.G. also showed an elevated glycine clearance but the plasma glycine was higher than that of any of the normal subjects studied here and above the normal range found by others with the same method (Evered, 1956; Stein & Moore, 1954). The tubular reabsorption of glycine by V.C. and E.G. was estimated to be 90 and 92% respectively, both somewhat lower than the range of 95–98% found in the normal subjects. These results strongly suggest a renal origin of the increased glycine excretion, but owing to the raised plasma glycine found in E.G. further analyses are needed to confirm this view.

Renal-tubular rickets with glycosuria

Although the presence of renal glycosuria in addition to renal phosphaturia in this patient might suggest that the tubular lesion was more extensive than in the adult patients with osteomalacia discussed above, the amino acid results show that the only notable departure from normal was a slight increase of glycine excretion and clearance with a normal plasma level. These changes are not quite so marked as in the two adults, but the reabsorption of glycine was estimated to be 92%, lower than in the normal subjects. The excretion and clearance of histidine were higher than in the normals but this may be a dietary effect. Apart from this only minor changes were seen.

Nutritional osteomalacia

Jonxis & Huisman (1953) have reported an increased excretion of amino acids by children suffering from classical rickets. Rickets in children due to simple vitamin D deficiency is now extremely rare in this country, but adults are occasionally seen who develop minimal signs and symptoms of osteomalacia due to unusual dietary habits (Dent, 1957). In the case studied here all the plasma amino acids were in the normal range but there was a two- to three-fold increase in the excretion of threonine, serine, glycine, alanine and histidine; glutamine excretion was probably similarly increased. There were small increases in the excretions of lysine and tyrosine and a large excretion of 1-methylhistidine. There was a very small excretion of proline, which is not detected at all in normal urine, and no peak was seen for cystine, indicating that the excretion of this amino acid was very much reduced. The increased clearance values mirror exactly the increases in excretion, and the estimated tubular reabsorptions of the affected amino acids were lower than normal, including glycine 86%, histidine 84%, serine 94% and threonine 97%. The urinary findings in this adult are very similar to those reported for rachitic children by Jonxis & Huisman (1953), and the other measurements show that this aminoaciduria is also of renal origin.

Adult hypophosphatasia

The particular point of interest in studying this patient was to obtain more information about the excretion of phosphoethanolamine, originally reported in children with the disease (McCance, Morrison & Dent, 1955; Fraser, Yendt & Christie, 1955) and subsequently confirmed in child and adult cases (Cusworth & Dent, 1956; Cusworth, 1958). The estimation of phosphoethanolamine was technically difficult, especially in plasma as the separation obtained between phosphoethanolamine and taurine on the column was not as good as that reported by Moore & Stein (1954*a*). A reasonably satisfactory analysis was obtained on urine giving an excretion rate of phosphoethanolamine of 140 μg./min./1·73 m.^{2}, which corresponds to a 24 hr. excretion of about 200 mg. A very small peak was obtained in plasma and, owing to the uncertainty of its measurement due to the inadequate resolution and low colour yield with the ninhydrin reagent, the fractions containing the peak were combined and their total phosphorus content was estimated by the method of Berenblum & Chain (1938) after digestion with potassium sulphate and sulphuric acid. This procedure was carried out for two separate column analyses of the same plasma sample but the agreement of results was poor, the ninhydrin measurements giving 0·32 and 0·49 mg./100 ml. and the phosphorus estimations 0·28 and 0·17 mg./100 ml. as phosphoethanolamine. An accurate clearance cannot be calculated from such results, but they indicate that the clearance is high, probably greater than 30 ml./min. Apart from the phosphoethanolamine and a high taurine excretion, the excretion rates of most of the amino acids were within normal limits. Threonine and serine were lower than normal and there was a small excretion of proline. In the plasma, the threonine concentration was low, possibly accounting for the low excretion although the serine concentration was normal. The plasma-proline concentration was raised. The concentrations of other amino acids in plasma were normal and the renal clearances were normal. These results do not confirm a previous suggestion, based only on paper

chromatography, that the excretion of the common amino acids in this disease was a little lower than normal (Cusworth & Dent, 1956).

Chronic renal failure with secondary hyperparathyroidism and aminoaciduria

The plasma concentrations of several of the amino acids were lower than normal, possibly as a result of their continual heavy loss in the urine. The excretion rates of most of the amino acids were raised two to ten times above normal. The most striking relative increases were in proline and ornithine, but in absolute amounts glutamine/asparagine, alanine, proline and glycine were excreted in greatest quantity. The renal clearances of most of the amino acids were increased several times above normal, and when they are compared with the estimated glomerular filtration rate of less than 10 ml./min. they are relatively very high indeed, and show the renal origin of the increased amino acid excretion. Approximate calculations showed that although the amino acid-filtered loads were greatly below normal owing to the low glomerular filtration rate, the fractions reabsorbed were much reduced, ranging from 60 to 80% for valine, leucine, ornithine, lysine and arginine to 7% for glycine. There was a gross reduction of tubular reabsorption of practically all the amino acids, so that in spite of the very low filtered loads, considerable amounts of amino acids were excreted. The acquired tubular defect in this case is similar qualitatively to the inborn defect found in cases of adult Fanconi syndrome discussed below.

Adult Fanconi syndrome

The plasma concentrations of most of the amino acids were normal, but glycine was slightly raised and proline markedly raised above normal. The excretion of nearly all the amino acids was grossly raised, ranging from a tenfold increase of threonine, serine and glycine to a 100-fold increase of valine. The indicated increase in glutamine/asparagine was even greater but this value is approximate owing to decomposition of glutamine on the column. Earlier paper-chromatographic studies (Dent, 1947) suggest that this increase is largely due to glutamine. There were large excretions of proline and ornithine, neither of which is detected in normal urine. In absolute terms, glutamine/asparagine, alanine, proline and glycine were excreted in greatest amount. The renal clearances of all the amino acids were considerably raised and should be compared with the estimated glomerular filtration rate of about 30 ml./min. The clearances of serine and cystine approached the filtration rate. Calculation of tubule functions indicated that, in spite of the considerably reduced filtered loads, only taurine was reabsorbed by more than 50%, whereas the reabsorption of serine and cystine was very low at 8%. These results confirm the renal origin of the aminoaciduria in the adult Fanconi syndrome and show the gross and generalized nature of the defect.

Cystinosis

The plasma concentrations of most of the amino acids were slightly lower than normal in both children. The excretion of most of the amino acids was moderately raised, serine and glycine at two to three times normal showing a lesser increase than alanine, valine and cystine, which were five to ten times normal. The increases of proline and ornithine excretion were relatively large but not so marked as in the adult with Fanconi syndrome described above. In absolute amounts alanine, glycine, proline and glutamine/asparagine were excreted in greatest quantity. Although the amino acid-excretion pattern was very similar in both children to that of the adult patient, and to children in earlier stages of the disease (Dent & Harris, 1956), the increase was not so marked. The renal clearances of the amino acids in the two children were raised above normal but not to the same extent as in the adult patient, even allowing for their lower filtration rates. Estimates of tubule function showed that the filtered loads of all the amino acids were very low and that the tubules reabsorbed a relatively higher proportion of the load compared with the adult patient, although the absolute reabsorption of the individual amino acids (corrected for surface area) was of the same order as in the adult. These results confirm the suggestion of Dent & Fowler (quoted by Dent, 1954), based on total α-amino-nitrogen measurements, that the aminoaciduria in cystinosis is of renal origin. This is contrary to the claim of Bickel & Smellie (1952) and of Woolf (1951) that this aminoaciduria is due to an elevation of the plasma amino acids and is in accordance with the more guarded later report of Bickel (1955). Our results also indicate that in the later stages of the disease glomerular function declines more rapidly than tubular amino acid reabsorption. Filtered loads decrease whereas reabsorption probably undergoes little change, so that the total amount of amino acids excreted decreases. This decrease in amino acid excretion as uraemia increases, in later stages of the disease, has frequently been noted on paper chromatograms.

Hartnup disease

In the plasma, threonine and serine concentrations were less than normal and the glutamine/asparagine peak was smaller than seen in normal subjects. The low values may have resulted from the high urinary losses. The concentrations of

proline and lysine were slightly raised. The excretion of most of the amino acids was grossly raised but glycine was increased only twofold, cystine threefold and proline and ornithine were not detectable. Arginine excretion was probably raised only to a small extent. The largest peak in the urine analysis was that due to glutamine plus asparagine; paper chromatograms have shown that a large amount of asparagine is present in the urine in addition to glutamine. Histidine and serine excretions were also very high. The clearance values reflect these measurements and give very high values for serine, glutamine/asparagine, histidine and threonine. On the other hand the clearances of glycine and cystine were only two to three times normal, whereas those of proline and ornithine were normal. Tryptophan was not detected in the plasma; in the urine a small diffuse peak was seen in the tryptophan/arginine region, which it was not possible to identify by inspection. Indole chromatograms (Jepson, 1955) have shown that the excretion of tryptophan is increased in this disease and it is unfortunate that the instability of tryptophan under the conditions used here have prevented any measurements from being made. Estimates of tubule function underline the unusual nature of this gross renal aminoaciduria. Renal function as measured by all the usual tests is quite normal, but there is a gross defect of amino acid reabsorption of a highly specific nature. Although only 30–50% of the filtered load of some amino acids is reabsorbed, the reabsorption of glycine, cystine, lysine and possibly arginine is affected to a very much smaller extent, whereas that of proline and ornithine is normal.

Argininosuccinic aciduria

These measurements were carried out before the amino acid-like substance excreted in large amounts by the two patients of Allan, Cusworth, Dent & Wilson (1958) had been identified as argininosuccinic acid (Westall, 1958). A preliminary column analysis was carried out on a random sample of urine and revealed a moderately large peak immediately after leucine, followed immediately by a second extremely large peak. Paper chromatography of alternate fractions from the region of these peaks showed that the first peak to emerge consisted almost entirely of a substance found in small amount on paper chromatograms of fresh urine from these cases and considered to be a decomposition product of the main spot (see fig. 3 of Allan *et al.* 1958). The second large peak consisted of roughly equal amounts of the main spot seen on paper chromatograms of whole urine from the affected children and another spot seen only in badly stored and somewhat decomposed urine samples. It is beyond the scope of this paper to discuss the detailed chemistry of these various degradation products, and to obtain an approximate figure for the amount of material being excreted the colour values of both peaks found in the column runs were added together. This was felt to be justified as paper chromatograms of fresh urine and plasma had shown the presence of traces only of the degradation products arising on the ion-exchange column. It was assumed by Allan *et al.* (1958), who gave approximate estimates of the quantity of the unknown 'amino acid' excreted, that the colour yield in the ninhydrin reaction was equivalent to that of leucine and that the molecular weight was 150. The molecular weight is now known to be 290. A similar picture of two unknown peaks was found in the timed urine sample, in the plasma and in a sample of cerebrospinal fluid. The results from these analyses were also treated as indicated above. In this paper we are calculating the excretions on the basis of the now-known molecular weight of the compound but have still to assume as a first approximation that the ninhydrin colour yield is the same as that with leucine. A small correction may be necessary later when pure samples of argininosuccinic acid are available. The results for the argininosuccinic acid calculated in this manner were: cerebrospinal fluid 10·0 mg./100 ml., plasma 3·3 mg./100 ml., excretion 3·5 mg./min./1·73 m.², clearance 105 ml./min./1·73 m.². Westall (1958) has now explained the phenomenon of double peaks on the elution curve and of the triple spots on the chromatograms as being due to the formation of two cyclic anhydrides from the argininosuccinic acid. It is a fair assumption that their ninhydrin values are similar to that of argininosuccinic acid and that our technique of quantitation is therefore probably correct. We emphasize that any errors introduced as above cancel out in determining the renal clearance.

The plasma concentrations of the other amino acids are now published for the first time. Many are normal but glycine was raised, 3·6 mg./100 ml., the highest value encountered in this series. Tyrosine was also elevated, being twice as large as that in any of the other subjects studied here. On the other hand, valine, lysine, arginine and ornithine were all slightly lower than normal and no peak at all was seen for cystine. The urine results confirm the paper-chromatographic observations of Allan *et al.* (1958) in that threonine, serine and possibly glutamine excretions were less than normal. The glycine excretion was at the low end of the normal range in spite of the elevated plasma concentration. Tyrosine excretion was slightly raised but histidine was very low indeed, only one-tenth of that seen in normal subjects. Excretion values for many of the neutral amino acids in the middle

of the chromatographic run were not obtained owing to the technical difficulties imposed by the presence of the very large abnormal peaks. All of the renal clearances for which values were obtained (except the argininosuccinic acid) were very low, notably glycine, serine and threonine; the histidine clearance was very low. These minor changes of other amino acids are difficult to explain but hardly alter the outstanding fact that the only gross abnormality concerns argininosuccinic acid.

DISCUSSION

The results confirm the renal nature of most of the conditions of aminoaciduria studied by our clearance techniques. The quantitative figures illustrate in addition the highly specific nature of some of the excretion patterns.

The abnormal amino acid excretion in argininosuccinic aciduria and in hypophosphatasia is of the 'overflow' type, but differs from that which occurs in phenylketonuria, liver disease and the recently described 'maple-syrup urine' disease (Westall, Dancis & Miller, 1957). In the last-named diseases there is an increased plasma concentration, and hence filtered load, of amino acids so that increased amounts are able to leak past the renal tubules into the urine, even though the tubules reabsorb a large part of the increased load. However, argininosuccinic acid, and probably phosphoethanolamine also, does not appear to be normally reabsorbed by the renal tubules. There is no renal threshold for them so that like inulin they are excreted in the urine whenever present in the plasma, even at low concentration.

The clearance of argininosuccinic acid is approximately equal to the filtration rate, indicating that little or no tubular reabsorption occurs. The calculated clearance of phosphoethanolamine in the patient with hypophosphatasia was not as high as the filtration rate, but owing to the low plasma concentration and the difficulty of its measurement, the true clearance may be somewhat higher than the value obtained here. If the excretion of phosphoethanolamine in hypophosphatasia were of renal origin it should be present in normal and hypophosphatasia plasmas at about the same concentration; it is not detected in normal urine, so if present in normal plasma it must normally have a very low clearance. This seems unlikely as intravenously administered phosphoethanolamine is very rapidly excreted by normal individuals (McCance, Fairweather, Barrett & Morrison, 1956), indicating that it has in fact a high clearance. The available evidence therefore suggests that phosphoethanolamine undergoes little or no tubular reabsorption in the normal state or in hypophosphatasia.

The present studies and those of Evered (1956) suggest that β-amino*iso*butyric acid may also be excreted in this manner. The excretion of large amounts of this amino acid by normal individuals (Crumpler, Dent, Harris & Westall, 1951) was initially considered to be due to defective renal-tubular reabsorption of the compound. Evered (1956) obtained an approximate clearance of β-amino*iso*butyric acid in one of these individuals which approached the filtration rate, but found that the plasma concentration in several subjects who excreted only small amounts of the amino acid was too small to be measured. This latter observation is confirmed in the present series of analyses; in the majority of subjects with normal renal function no peak was obtained in the β-amino*iso*butyric acid region of the plasma analysis. Consideration of the β-amino*iso*butyric acid excretion of these subjects shows that the clearance must have been high to hold the plasma concentration below the limit of detection. On the other hand, in three subjects (E.Hu., J.J. and G.J.) with severe renal damage small but measurable peaks were present in the plasma as if the β-amino*iso*butyric acid had been dammed back in a similar manner to creatinine and urea, and in each case the clearance of the amino acid was similar to the creatinine clearance Feeding experiments have shown that β-amino*iso*butyric acid is rapidly excreted in the urine when ingested by normal subjects (Evered & Westall, quoted by Dent & Senior, 1955), again suggesting a high rate of renal clearance.

In addition to these examples, a similar mechanism appears to operate in the excretion of homogentisic acid (Neuberger, Rimington & Wilson, 1947) and porphobilinogen (Goldberg & Rimington, 1954). Many of these substances have something in common in that they appear to be normally exclusively intracellular, that is they are not transported about the body in the plasma, each cell being responsible for its own supply of the compound. Many substances such as glucose and most amino acids are transported in the plasma and if they were continually excreted in large amounts, owing to a low renal threshold, it would represent a serious loss to the body's metabolic economy. On the other hand a low or non-existent renal threshold is of no consequence with substances normally confined to the cell interior, and the arrangement only becomes conspicuous and possibly disadvantageous when for some reason they enter the plasma. If the unusual excretion of substances such as argininosuccinic acid and phosphoethanolamine is not due to a reduction of tubular reabsorption it is necessary to look for an extrarenal cause for the increased plasma concentration. β-Amino*iso*butyric acid is normally present in plasma at very low concentration; the increased

excretion shown by certain individuals is thus presumably also due to an increased plasma concentration. A possible cause is a metabolic block in which, due to an enzymic defect, the concentration of the non-metabolized substance increases in the cell and so leaks into the plasma. This may be so in hypophosphatasia, where there is a demonstrable deficiency of an enzyme or enzymes which can hydrolyse phosphoethanolamine. Another possibility is an alteration of the permeability of the cell membrane so that the substance enters the plasma even though the intracellular concentration is not increased. These two mechanisms are analogous to overflow and renal aminoaciduria, but operating at the level of the cell in question, which is not necessarily in the kidney.

Another point which arises from these studies is the not infrequent association of renal aminoaciduria and impaired vitamin D and phosphate metabolism. This has also been noted by Harrison & Harrison (1957). Examples studied here include renal-tubular oesteomalacia, simple vitamin D deficiency, chronic renal failure with secondary hyperparathyroidism and the Fanconi syndrome. A similar association occurs in the syndrome of Lowe, Terrey & MacLachlan (1952), and further evidence has been obtained from an extended study of a patient with this syndrome in which there was a marked reduction of amino acid excretion during treatment with vitamin D (J. P. Bound, D. C. Cusworth, C. E. Dent and J. M. Smellie, in preparation). A possible common factor in this group of diseases is the apparent absence of vitamin D action, due either to simple deficiency or to its failure to act when present in normal amounts. Another possibility is that parathyroid-gland hormone is involved, but in any case the mechanism does not seem to be a simple one such as a common reabsorption pathway for phosphate and amino acids. Further study of the renal handling of amino acids in various vitamin D states is needed before this association can be interpreted.

SUMMARY

1. Ion-exchange chromatography has been used to estimate the free amino acid content of timed urine and simultaneous plasma samples collected under fasting conditions from healthy adults and from patients with abnormal amino acid excretion.

2. Calculation of renal clearances confirms that 98% or more of the filtered load of most amino acids is normally reabsorbed by the renal tubules.

3. The increased glycine excretion by some cases of renal-tubule osteomalacia and the aminoaciduria of adult vitamin D deficiency are of renal origin. The depression of tubular amino acid reabsorption in these cases is small and limited to one or a few amino acids.

4. In the renal aminoaciduria of Hartnup disease, adult Fanconi syndrome, cystinosis and in a patient with acquired renal damage with a tubular lesion similar to that in the Fanconi syndrome, the reabsorption of many amino acids was very much reduced, being as low as 10% of the filtered load in some cases.

5. A form of overflow aminoaciduria is described which involves amino acids not normally detectable in plasma, and for which no renal reabsorptive mechanism exists. Argininosuccinic acid is excreted in this manner and the excretion of phosphoethanolamine in hypophosphatasia probably has a similar origin. β-Amino*iso*butyric acid, normally present in plasma in very low concentration, is also possibly excreted in this way.

APPENDIX

Clinical details of subjects studied

Normal adults. These were healthy laboratory workers with no history of renal disease.

Osteomalacia with increased glycine excretion. V.C. and E.G. were patients with Type I renal-tubular osteomalacia (Dent, 1952*b*) in which the tubular defect described is of diminished reabsorption of phosphate only. The increased glycine excretion in these patients, which might indicate a further tubular defect, was reported by Dent & Harris (1956) and their 24 hr. urine amino acid excretions were measured by Evered (1956). The increased glycine excretion has not been found in all Type I cases but only in those that present in adolescence or early adult life and develop severe bone disease. Both V.C. and E.G. previously had severe osteomalacia, which, however, responded well to treatment with large doses of vitamin D. E.G. had been off vitamin D for 1 month owing to a previous mild intoxication. The clinical investigations suggested a slight continuing vitamin D action at the time the clearance was done. V.C. at the time was responding fully to a large daily dose (5 mg.) of dihydrotachysterol given as AT 10.

Renal-tubular rickets with glycosuria. S.K., from the age of 18 months, had been noted to have rickets not cured by normal doses of vitamin D and only slowly responsive to very large doses. He otherwise had good general health with no evidence of renal or intestinal disease. On further study recently he was shown to have a mild renal glycosuria and high phosphate clearance (Type II of Dent, 1952*b*). There was no aminoaciduria detectable by paper chromatography. When the clearances were determined he had had no vitamin D treatment for some months previously.

Nutritional osteomalacia. L.R. had for many years chosen to live on an unusual diet from which milk, butter, cheese, eggs and fish had been rigidly excluded. Her vitamin D intake was estimated to be less than 20 units/day. She suffered from severe bone pains and although there was no radiological evidence of bone changes, biochemical findings were typical of osteomalacia with plasma calcium 8·8 mg., phosphorus 2·0 mg. and alkaline phosphatase 20 King–Armstrong (King & Armstrong, 1934)

units/100 ml. She responded well to treatment with dihydrotachysterol. The amino acid studies were carried out before treatment was commenced. Her clinical history was briefly summarized by Dent (1957).

Adult hypophosphatasia. The full clinical description of this patient, M.P., has been given by Bethune & Dent (1960). She had suffered from 'rickets' in childhood from which a slow recovery was made. She was well from 20 years of age until she was 34. During the next 6 years she suffered increasing pains in her feet, back and lately in both hips, so that she had become severely crippled. X-ray examination showed unhealing fractures in the middle of both femurs. All routine biochemical investigations were normal except for a constantly low plasma alkaline phosphatase level. Paper chromatography, however, showed a constant large excretion of phosphoethanolamine in the urine. The identity of the phosphoethanolamine has been confirmed by isolation and analysis from this patient's urine (Cusworth, 1958). No treatment was being given when the amino acid clearances were determined.

Chronic renal failure with secondary hyperparathyroidism. E.Hu. had long-standing renal damage which was first noted during her third pregnancy 17 years previously. During the last 2 years increasing bone pains and muscular weakness heralded the gradual development of secondary hyperparathyroidism. More recently the renal failure and radiological changes in her bones had become more severe. On admission to hospital blood urea was 168, plasma calcium 10·3 and plasma phosphorus 10·2 mg./100 ml., alkaline phosphatase was 23 King–Armstrong units, standard urea clearance was 11% of normal.

X-ray examination showed gross generalized arterial calcification as well as advanced hyperparathyroidism. Rather unexpectedly her urine paper chromatograms showed a definite aminoaciduria. Her amino acid clearances were determined soon after admission while no treatment was being given. She died 2 months later in uraemia. At post-mortem examination gross secondary enlargement of the four parathyroid glands was found as well as the other expected changes.

Adult Fanconi syndrome. The clinical history of this patient, M.Y., was described in full (case 1) by Milne, Stanbury & Thomson (1952). She had presented herself with typical osteomalacic symptoms developing 8 years previously with subsequent attacks of weakness due to 'low-potassium attacks'. She showed all the clinical and biochemical signs of the so-called 'adult Fanconi syndrome' (Dent & Harris, 1956) and had made an excellent clinical response to vitamin D and alkalis.

At the time the amino acid studies were carried out the patient had become seemingly 'resistant' to the continuing calciferol and alkali therapy and balance studies showed that she was in a virtually untreated state. She subsequently responded well to dihydrotachysterol and alkali. She has continued symptom-free to the time of writing (June 1959).

Cystinosis. J.J. and G.J. were brother and sister both showing the typical clinical and biochemical features of this disease. At the time of the amino acid studies both were in uraemia but with the rickets healed and in fair general health. They were each receiving 1·25 mg. of calciferol and 8 g. of sodium bicarbonate daily. Both children died 1 year and 6 months later respectively, with uraemia and hypertensive failure.

Hartnup disease. E.Ha. was case 1 of Baron, Dent, Harris, Hart & Jepson (1956). He had had the typical attacks with pellagra-like rash and cerebellar ataxia. In addition he showed always a gross aminoaciduria of unvarying pattern, an increased excretion of indole derivatives and other features which these authors have described. At the time of the clearance determinations he was in good general health.

Argininosuccinic aciduria. K.R. was case 2 of Allan *et al.* (1958). He was severely mentally retarded and was still manifesting the constant gross excretion of an amino acid-like compound that these authors described and which has now been identified by Westall (1958) as argininosuccinic acid. He was on a normal diet and having no treatment at the time of the clearance determinations.

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Studies on the Binding of ^{65}Zn by Equine Erythrocytes *in vitro*

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The administration of zinc to rabbits has been found to cause an increase in the zinc content of the erythrocytes unaccompanied by an increase in the content of carbonic anhydrase (Berfenstam, 1952). In humans also, the injection of ^{65}Zn was found to result in an uptake of about 10% of the injected element by the entire blood-cell mass in approximately 10 min. The specific activity of the erythrocytes continued to rise progressively until ultimately it exceeded by two- to three-fold that of the plasma (Gibson, 1953). Sheline, Chaikoff, Jones & Montgomery (1943) have shown that, in dogs, 10 hr. after the injection of ^{65}Zn all of the isotope is found in the erythrocyte and none in the plasma, even though initially the plasma had bound an appreciable amount of the isotope. The erythrocytes therefore have a marked capacity to take up ^{65}Zn even from a medium such as plasma, for instance, where many physiologically important metal-binding ligands are present. However, little is known about the process except that when the cells are incubated *in vitro* with ^{65}Zn they take up the isotope and fix it in a form that is stable to exhaustive washing with sodium chloride solution (Gibson, 1953; Tupper, Watts & Wormall, 1951, 1952), and that carbonic anhydrase is not involved in this binding (Tupper *et al.* 1952). In this paper, certain features of the binding of ^{65}Zn by washed erythrocytes *in vitro*, and the influence of other cations, metabolic inhibitors and some amino acids on this uptake, are presented.

EXPERIMENTAL

Erythrocyte suspension. Horse blood cells, freshly obtained, were used in all experiments. The cell suspension was prepared from citrated blood by separating the erythrocytes from the plasma and repeatedly washing the cells with large amounts of iso-osmotic NaCl soln. (0·9%) until free of citrate and plasma constituents. The suspension was then diluted with 0·9% NaCl so that the final suspension had a total solids content of 170 mg./ml. This suspension thus had approximately the same erythrocyte content as whole blood, since the latter contains 17 g. of haemoglobin/100 ml. The cells were invariably used with minimum delay after preparation of the above suspension. It was found that some differences existed between different samples of blood in zinc-binding capacity. Hence, in each set of experiments, the same sample of blood was employed.

Incubation procedure. Erythrocyte suspension (1 ml.) and 1 ml. of ^{65}Zn salt solution (preparation and activity given below) were incubated in a total volume of 4 ml. The rest of the volume was 0·9% NaCl when the erythrocytes were incubated with zinc alone, or of a suitable solution in 0·9% NaCl of any compound(s) whose influence on zinc uptake was to be tested. Under these conditions, the dilution of ^{65}Zn in the incubation mixture was such as to provide a final concentration of zinc of 0·25 mM.

When the effect of metal ions was studied the procedure was somewhat different. Erythrocyte suspension (1 ml.), 1 ml. of 4 mM (or where desired 40 mM) solution of the respective metal ion prepared in 0·9% NaCl and 1 ml. of 0·9% NaCl were first incubated for 2 hr. Thereafter, 1 ml. of ^{65}Zn salt solution was added and the incubation continued for another 2 hr. This procedure was adopted to eliminate, as far as possible, any differences in the final

result brought about by probable differences in the rates of influx of the metal ions into the erythrocytes. Copper was used at much lower concentrations (10 and 100 μM final concentration), since it was found to be a very powerful lysing agent at higher levels.

2:4-Dinitrophenol, glutathione, *p*-chloromercuribenzoate and cyanide were also tested at mM concentration in the medium, and erythrocytes were pre-incubated with the compounds in a manner similar to the above, except that the pre-incubation period was 1 hr. Several amino acids were also tested with a pre-incubation period of 30 min. Preliminary experiments, carried out with a view to determine the optimum pre-incubation periods for each of these classes of compounds, revealed that they had different rates of migration and lead to the choice of the periods. These were optimum for the present investigation.

In all cases, all solutions were prepared in 0·9% NaCl, with suitable precautions to eliminate contamination from other trace elements, and adjusted to pH 5·5 before use. Incubations were carried out in triplicate, in Pyrex test tubes of 20 ml. capacity, which were kept in a metal test-tube rack in a Dubnoff metabolic incubator and shaken at room temperature (30°).

For determination of zero-time uptake of zinc by erythrocytes, 1 ml. of erythrocyte suspension was rapidly blown into 1 ml. of ^{65}Zn salt solution and 2 ml. of 0·9% NaCl, mixed by rapid shaking for a few seconds and immediately centrifuged at 100 *g*. After 2 min., the supernatant was quantitatively separated and the cells were processed for determination of ^{65}Zn.

In the experiments where the effect of pH on the uptake of ^{65}Zn by the cells was studied, 1 ml. of the erythrocyte suspension was taken in test tubes, centrifuged to pack the cells and the supernatant was pipetted out. ^{65}Zn in 0·9% NaCl, adjusted to the desired pH, was then added to bring the final volume to 4 ml. and the usual ^{65}Zn concentration. The influence of pH was studied at pH 4·0, 5·5 and 6·0. With whole erythrocytes, a pH of less than 4·0 could not be used since it was found to induce agglutination and lysis. Similarly, under the conditions of the present investigation, a partial precipitation of zinc was found to occur beyond pH 6·0 and hence uptake of ^{65}Zn was not studied at higher pH.

Radioactive technique. Radioactive zinc was purchased from The Radiochemical Centre, Amersham, Bucks, and had a specific activity of 60 mc/g. In one experiment (electrophoresis experiment, Fig. 2), zinc with a higher specific activity (100 mc/g.) was also employed. The zinc solution, as obtained, was $^{65}ZnCl_2$ in dilute HCl and was diluted with 0·9% NaCl and adjusted to pH 5·5 before use. The incubation mixture always contained about 9×10^3 counts/min., under the conditions of counting. The exact values in each experiment were not always identical since the experiments were performed during the course of a few months, and hence the actual number of counts has been indicated wherever necessary. However, the actual zinc concentration was constant in the incubation mixture, being 0·25 mM.

In all experiments, immediately after the termination of incubation with ^{65}Zn, the incubation mixture was centrifuged at 2500 rev./min. for 5 min. to stop uptake and to separate the cells from the unabsorbed ^{65}Zn. The supernatant was then removed by means of a glass tube with a fine nozzle and a rubber teat, without disturbing the cells. A portion (5 ml.) of 0·9% NaCl was then added, the cells were shaken to attain uniform suspension and again centrifuged as before; the 0·9% NaCl was discarded. The procedure was repeated four times in all. Preliminary tests showed that this procedure did not remove zinc taken up by the cells themselves. Control tubes were always run in the same manner and all erythrocyte samples were subjected to the centrifuging process at the same time so that the values were strictly comparable.

Radioactivity determinations of erythrocyte samples were performed by lysing the samples and making up to 10 ml. with water, in a Veall liquid counter (M6M, 20th Century Electronics Ltd.) attached to a Panax scaler of the decade type (Panax Equipment, Mitcham, Surrey). With solid samples, a weighed amount (not exceeding 10 mg.) was uniformly dispersed in a steel planchet and radioactivity measured with the aid of a bell-type Geiger counter (G.E.C. Counter, mica end-window, 2·1 mg./cm.^{2}) attached to the Panax scaler. All samples were counted such that the relative counting error was within $\pm 2\%$. No self-absorption corrections have been made.

Electrophoresis of erythrocyte proteins. In one experiment it was sought to associate the major part of the radioactivity taken up by the erythrocytes with the haemoglobin by paper electrophoresis of the erythrocyte proteins. For this purpose 1 ml. of the erythrocyte suspension was mixed with 1 ml. of ^{65}Zn salt solution (100 mc/g.) for a period of 4 hr. The cells were then washed with 0·9% NaCl until completely free of unabsorbed zinc and were lysed with ether. Water (0·5 ml.) was added, the haemolysate was centrifuged at 100 *g* to remove debris and two portions of 100 μl. were removed. In one of the portions the radioactivity was determined as before. A duplicate portion was then spotted on Whatman no. 1 filter paper and electrophoresis conducted for 3 hr. at 180 V and 5·5 mA in an Arthur Thomas Co. Paper Electrophoresis Apparatus, in 0·05 M-veronal buffer. After electrophoresis, the strip was removed, the position of the haemoglobin band was noted and the strip cut transversely into pieces 1 cm. wide. The ^{65}Zn from each strip was eluted by suspending it in 10 ml. of N-HCl in a test tube for 1 hr. with intermittent shaking. A few trials conducted as checks showed that this procedure quantitatively eluted the radioactivity from the paper. After centrifuging, the solution was then counted as usual in the liquid counter.

Isolation of haemoglobin. In a second experiment, cells after having been allowed to take up ^{65}Zn were lysed by adding a little ether, followed by a few drops of water. The haemolysate was then centrifuged to separate cell debris and the clear red supernatant was set aside in the ice-chest, to crystallize the haemoglobin, after adjusting the pH to 6·8 (Lemberg & Legge, 1949). After leaving the solution overnight, most of the haemoglobin was found to have crystallized in the form of big crystals and was separated by centrifuging. The supernatant was kept separate. The crystals were washed with the minimum amount of ice-cold water and the radioactivity in the haemoglobin was determined.

Dialysis and fractionation of erythrocyte proteins. The cells from the incubation mixture (1 ml. of erythrocyte suspension, 1 ml. of ^{65}Zn salt solution in 4 ml. volume, incubated for 2 hr.) were washed free of unabsorbed ^{65}Zn, lysed and the volume was made to exactly 5 ml. This haemolysate was dialysed against 40 ml. of double-distilled

water in a 50 ml. beaker overnight at 5°. The water was then changed and dialysis continued. Three changes of water were made in all, over a period of 72 hr., at the end of which the lysate and the dialysis water were removed for estimation of ^{65}Zn. The pooled dialysis water was evaporated to small volume, made up to 10 ml. and counted in the liquid counter. The haemolysate was also similarly treated and its radioactivity determined.

An experiment was performed in the following manner to determine broadly the distribution of radioactive zinc in erythrocyte proteins. A portion (10 ml.) of erythrocyte suspension was incubated with ^{65}Zn, under conditions similar to those described before, in 50 ml. centrifuge tubes for 2 hr. After washing with 0·9 % NaCl the cells were lysed and acetone was added slowly, with stirring, in an ice bath, to precipitate proteins. After the required concentration of acetone had been reached, the tubes were tightly plugged and set aside, overnight, in a refrigerator to complete precipitation. The precipitate was separated by centrifuging, washed twice with acetone of the same concentration and finally dried by washing with pure acetone. It was then dried to constant weight *in vacuo* and its ^{65}Zn content determined with the Geiger counter. As the primary purpose in these studies was to find out whether there were any marked variations in the specific activities of erythrocyte proteins, the fractionation was conducted in four steps and the proteins separating in the range of acetone concentration 0–25 %, 25–50 % and 50–75 %, and in the supernatant from the last stage, were tested. ^{65}Zn taken up by the cells was quantitatively precipitated when the acetone concentration was 75 %. Advantage was taken of this observation in studying the stability of bound ^{65}Zn to ethylenediaminetetra-acetate (EDTA). For this purpose, erythrocytes were incubated with ^{65}Zn for 2 hr. as usual, washed, lysed and reincubated with 4 ml. of 10 mM-EDTA at pH 5·5. At the end of the requisite incubation period, acetone was added to 75 % concentration and protein separated as above. The supernatant was evaporated, made up to 10 ml. in 75 % acetone and counted. Under these conditions, the radioactivity in the 75 % acetone supernatant represented the ^{65}Zn split off from the cell proteins by EDTA. The zinc in the ^{65}Zn used at the levels employed was found to be completely soluble in 75 % acetone, both in the presence and in the absence of EDTA.

RESULTS

In Fig. 1 is shown the uptake of ^{65}Zn by intact equine erythrocytes as a function of the time of incubation. The uptake of ^{65}Zn is more rapid in the first 2 hr. but thereafter slows down and, eventually, at the end of 4 hr. the uptake ceases. However, even at this time, Fig. 1 reveals that the ^{65}Zn taken up constitutes only about 30 % of the total present in the incubation mixture. To find out whether this represented the maximum that could be taken up by the cell proteins, the same amount of the cells (1 ml. of erythrocyte suspension) was lysed and allowed to take up ^{65}Zn under otherwise identical conditions. Uptake was stopped by adding acetone to 75 % concentration. When this was done it was found that the entire amount of added ^{65}Zn ($9{\cdot}23 \times 10^3$ counts/min.) was taken up by the erythrocyte proteins within 1 min.

In a separate study, the effect of acetone on uptake of ^{65}Zn by the erythrocyte proteins was determined as follows, with haemolysate instead of intact cells. The haemolysate used for the purpose was made by taking a measured amount of the final erythrocyte suspension, centrifuging to pack the cells, pouring off the supernatant, lysing with water and making up once again to the original volume. Hence, in protein content, 1 ml. of haemolysate was exactly equivalent to 1 ml. of cell suspension. Haemolysate (1 ml.) was incubated with 1 ml. of ^{65}Zn salt solution (9×10^3 counts/min.) and 2 ml. of 0·9 % NaCl for 2 hr., and the protein was precipitated with acetone to 75 % concentration. In this case, all the ^{65}Zn added was found to be bound to protein. Another portion of haemolysate (1 ml.) was incubated with ^{65}Zn as before and was then dialysed against double-distilled water for 96 hr. to remove all unbound ^{65}Zn. The haemolysate was counted after dialysis and the ^{65}Zn uptake value compared with the corresponding value obtained by the normal procedure outlined before. In one typical experiment, the mean values obtained were, for whole erythrocytes, 1903 counts/min. and for the haemolysate, after dialysis, 5227 counts/min.

These results and those presented in Fig. 1 together indicate that the uptake of ^{65}Zn by the whole cells is much less than the total zinc-binding capacity of the intracellular proteins. The lack of uptake of ^{65}Zn by erythrocytes beyond 4 hr. is presumably due to a cessation of zinc influx into the cell interior beyond this period.

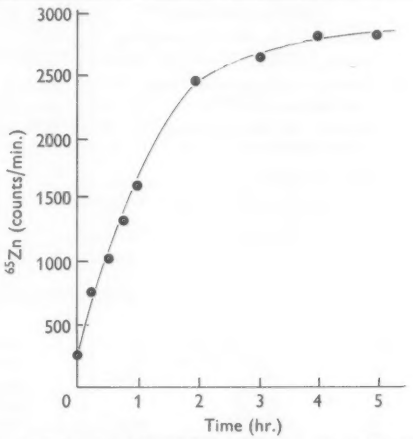

Fig. 1. Rate of uptake of ^{65}Zn by erythrocytes (total ^{65}Zn in the incubation mixture: $9{\cdot}23 \times 10^3$ counts/min.). For experimental details see text.

The above results also reveal that when the erythrocyte proteins are treated with acetone in the presence of ^{65}Zn the zinc-binding capacity is apparently increased. However, acetone was found not to have any effect on zinc already bound to protein.

The stability of bound ^{65}Zn to dialysis was then examined as described earlier and the results obtained are shown in Table 1. It can be seen that the zinc taken up by the cells is entirely bound to protein and that there is little free ionic zinc inside the erythrocyte.

In Table 2 is shown the distribution of ^{65}Zn in the several protein fractions of erythrocytes. The results show that the bulk of the radioactivity is associated with haemoglobin, a conclusion supported by (*a*) the electrophoresis pattern represented in Fig. 2 and (*b*) the ^{65}Zn found in the haemoglobin separated by crystallization after it had taken up the isotope; 85·3% (mean value from duplicates) was found in the haemoglobin.

The pH of the incubation medium was also found to influence uptake of ^{65}Zn by erythrocytes. In two sets of experiments carried out, the average uptake values at pH 4·0, 5·5 and 6·0 were 1108, 1904 and 4176 counts/min. respectively.

The influence of several metabolic inhibitors and complex-forming agents on the uptake of ^{65}Zn by erythrocytes is shown in Table 3. The absence of appreciable inhibition by cyanide and dinitrophenol would appear to indicate the possible absence of an energy requirement for uptake of ^{65}Zn. The considerable increase in the uptake in the presence of cyanide is somewhat unexpected. The explanation for such behaviour is probably associated with the greater permeability of the zinc cyanide complex, since the limiting step in the uptake process is the influx of zinc into the intracellular space. Further, subsequent experiments showed that the effect was absent when haemolysate was used instead of whole cells, supporting such a view.

The effect of EDTA on the uptake and the stability of bound ^{65}Zn by erythrocytes was studied in two ways, and the results are shown in Table 4. It can be seen that in the presence of EDTA, no ^{65}Zn is taken up and ^{65}Zn bound to erythrocytes is removed relatively slowly by subsequent treatment with EDTA (Table 4). It seems likely that EDTA (Foreman & Trujillo, 1954) and

Table 1. *Stability of erythrocyte-bound* ^{65}Zn *to dialysis*

For conditions of incubation and dialysis see text.

Expt. no.	Experimental details	Uptake of ^{65}Zn (counts/min.)*
1	Standard ^{65}Zn used in incubation	9012
2	Blank (of the liquid counter used)	14
3	Activity in erythrocytes after incubation with ^{65}Zn	2540
4	Activity of lysed erythrocytes after dialysis	2510
5	Dialysis water	17

* Values are corrected for background.

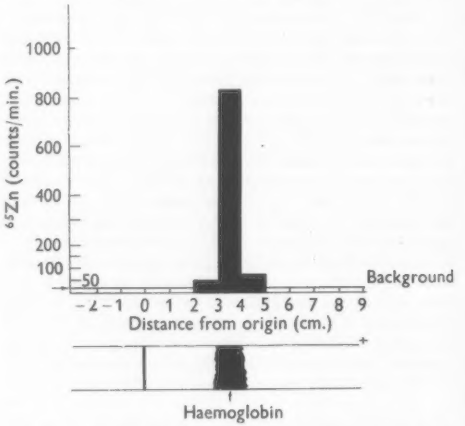

Fig. 2. Distribution of ^{65}Zn in erythrocytes (100 μl. of haemolysate of erythrocytes with total count of 913 counts/min. spotted on paper). Electrophoresis was carried out as described in the text.

Table 2. *Distribution of* ^{65}Zn *in erythrocyte proteins*

For conditions of incubation and fractionation see text.

Fraction no.	Range of acetone concentration (%)	Colour of protein	Total wt. of ppt.	^{65}Zn content (counts/min.)*	
				Total	Content/mg.
I	0–25	Pale rose†	1·8 mg.	118	65·5
II	25–50	Rose red	1·7 g.	24 610	14·5
III	50–75	White	3·3 mg.	46	13·9
IV	Supernatant from III	—	—	Nil	Nil

* Total ^{65}Zn added at the incubation stage was $11{\cdot}5 \times 10^4$ counts/min.
† This fraction included stromata and cell debris.

zinc–EDTA complex do not penetrate the erythrocyte. Another important feature is that EDTA splits off bound ^{65}Zn only very slowly, the process going to completion only after somewhat more than 2 hr.

Table 5 summarizes the effect of other cations on the uptake of ^{65}Zn by erythrocytes: among the cations tested, only Fe^{3+} and, to a much smaller extent, Cu^{2+} ions are definitely inhibitory to the process of uptake. The concentration of Cu^{2+} ions in these experiments was one-hundredth of that of Fe^{3+} ions. The influence of these cations was also studied with the haemolysate instead of whole erythrocytes by the technique described above. Only Fe^{3+} was inhibitory, to the extent of 25·2% (control, 2560 counts/min.; with 3 mM-Fe^{3+}, 1928 counts/min.; ^{65}Zn added was $9{\cdot}7 \times 10^3$ counts/min.).

Christensen & Riggs (1956) have offered evidence for the beneficial influence of chelate formation on the transfer of amino acids (and consequently of the metal ions associated with such complexes) into living cells. It was thought of interest therefore to find out whether amino acids have any influence on the uptake of ^{65}Zn by erythrocytes. The results of such a study are shown in Table 6. It will be evident that several of the amino acids are inhibitory. However, none of the amino acids was inhibitory to the uptake of ^{65}Zn by haemolysate.

Table 3. *Influence of some metabolic inhibitors and related compounds on uptake of* ^{65}Zn *by erythrocytes*

For conditions of incubation see text. Concentration of the tested compounds in the incubation mixture was 1 mM.

Compound tested	Uptake of ^{65}Zn (counts/min.)*
1 Control (none)	2443
2 Sodium cyanide	7287
3 2:4-Dinitrophenol	1801
4 *p*-Chloromercuribenzoate	2566
5 Glutathione	224

* ^{65}Zn added: $8{\cdot}52 \times 10^3$ counts/min.

DISCUSSION

The results obtained in this investigation have revealed several features of zinc uptake by whole erythrocytes *in vitro*. They are in agreement with the findings of Tupper *et al.* (1952) that the ^{65}Zn taken up is fairly stable and is entirely bound to erythrocyte protein. It can be seen that this uptake is also markedly influenced by the pH of the incubation medium, showing that the presence of H^+ ions in excess is detrimental to the process. From the data of Table 2 it would appear that there is little free Zn^{2+} in the intracellular space, since the 75% acetone supernatant, which is free of protein, is also free of radioactivity. Even though the uptake of ^{65}Zn is increased when the haemolysate is incubated with ^{65}Zn in the presence of acetone, acetone by itself was found to have no effect on ^{65}Zn taken up by erythrocyte proteins. Thus the distribution of ^{65}Zn among the erythrocyte proteins as shown in Table 2 is essentially representative of the capacity of the several proteins in this respect. Doubts have earlier been expressed (cf. Gibson, 1953) whether the ^{65}Zn taken up by the erythrocytes represented that bound to the cell membrane itself or whether it entered the cell and was bound inside. From

Table 4. *Influence of ethylenediaminetetra-acetate on* ^{65}Zn *binding by erythrocytes*

A: 1 ml. of erythrocyte suspension was incubated with EDTA and ^{65}Zn (9×10^3 counts/min.) for 2 hr., the cells being thereafter processed for determination of uptake as usual. B: erythrocytes, after having been allowed to take up ^{65}Zn as in A, but without EDTA, were lysed and reincubated with EDTA at pH 5·5; after incubation, the protein was precipitated by adding acetone to 75% concentration, and the acetone supernatant counted to determine the amount of zinc split off by EDTA.

	Content of ^{65}Zn (counts/min.)	
	In erythrocytes	In acetone supernatant
A. Influence of EDTA on ^{65}Zn binding		
(1) Control (no EDTA)	2582	—
(2) With 1 mM-EDTA and ^{65}Zn	43	—
(3) With 10 mM-EDTA and ^{65}Zn	19	—
B. Effect of EDTA on ^{65}Zn bound by erythrocytes		
(1) Control	2560	Nil
(2) Incubated with 10 mM-EDTA		
(i) For 2 hr.	871*	1689
(ii) For 3 hr.	6*	2554
(iii) For 4 hr.	10*	2550

* Values calculated by difference from total uptake and the radioactivity of the acetone supernatant.

Table 5. *Influence of metal ions on* ^{65}Zn *uptake by erythrocytes* in vitro

For conditions of incubation see text.

Expt. no.	Metal ion tested and salt employed	Uptake of ^{65}Zn (counts/min.)* Concn. 1 mM	Concn. 10 mM
1	None (control)	2943	
2	Fe^{3+} ($FeCl_3$)	108	—†
3	Co^{2+} ($CoCl_2$)	2788	332‡
4	Mn^{2+} ($MnSO_4$)	3030	3114
5	Ca^{2+} ($CaCl_2$)	2942	2870
6	Cd^{2+} ($CdSO_4$)	3041	—†
7	Cu^{2+} ($CuSO_4$)	(2594)§	(1891)‖

* ^{65}Zn added in the incubation mixtures: 9×10^3 counts/min.
† Complete lysis.
‡ Agglutination of cells.
§ Cu^{2+} at 10 μM.
‖ Cu^{2+} at 100 μM.

Table 6. *Influence of amino acids on uptake of* ^{65}Zn *by erythrocytes*

The ^{65}Zn added was as in earlier experiments, but due to decay provided only 6×10^3 counts/min.

Expt. no.	Amino acid tested	Uptake of ^{65}Zn (counts/min.)
1	None (control)	1220
2	Histidine	124
3	Lysine	680
4	Glutamic acid	732
5	Leucine	802
6	Glycine	943
7	Arginine	972
8	Methionine	1000
9	Tyrosine	1185

Table 2 it may be inferred that little zinc is bound by the cell membrane itself, since fraction I (Table 2), which contains stromata and cell debris and comparatively little other protein matter, contains a negligible amount of the total radioactivity taken up by the cells, though the specific activity is higher than that of haemoglobin.

The uptake of ^{65}Zn reaches a maximum in 4 hr., and hence is comparatively faster than the uptake of iron in duck erythrocytes, which is known to require 6 hr. (Sharpe, Krishnan & Klein, 1952). However, the reason why the uptake of ^{65}Zn should normally fall short of the total binding capacity is not clear. As indicated by the unique effect of cyanide (Table 3) and the observed binding capacity of the haemolysate, the phenomenon is probably unrelated to a limited binding capacity or to a normal barrier to the influx of the metal ion. It is possible that continued exposure of erythrocytes to Zn^{2+} ions, under the conditions of the experiments, has an adverse effect on the cell membrane resulting in decreased permeability. The well-known protein-precipitating ability of Zn^{2+} ions, and the fact that exposure of erythrocytes to mM-concentration and above of Zn^{2+} ions was found to result in an immediate clumping of the cells, followed by lysis, support such a hypothesis.

During recent years, various investigations have been reported (Sharpe *et al.* 1952; Boiron, Paoletti & Tubiana, 1955; Fleckenstein, Gerlach & Janks, 1956; Saltman, Fiskin & Bellinger, 1956*a*; Saltman & Boroughs, 1958; Pal, 1958; Clarkson & Kench, 1958) on the capacity of erythrocytes, as well as cells from other tissues such as liver, to bind ions such as Fe^{3+}, Pb^{2+} and Mn^{2+}. A comparison of the results of the present investigation with some of the data for the other cations reveals several interesting points. As with liver tissue with ^{65}Zn (Saltman & Boroughs, 1958) and ^{59}Fe (Saltman *et al.* 1956*a*; Saltman, Fiskin, Bellinger & Alex, 1956*b*) the uptake of Zn^{2+} ions by erythrocytes appears to be largely a passive process. Further, erythrocytes seem to possess different binding properties for different cations in view of the differences exhibited in Pb^{2+} binding (Clarkson & Kench, 1958) and ^{65}Zn binding; with the former ion the binding appears to be reversible and up to 25% of the total is present at the cell membrane, whereas little zinc appears to be bound at the cell surface.

The effects of other cations on zinc uptake show the great degree of specificity involved in the binding of ^{65}Zn by the erythrocytes. It is difficult on the basis of available literature to say whether the various ions exhibit comparable rates of diffusion across the erythrocyte membrane. However, in view of the fact that the erythrocytes had been pre-incubated with the cations tested for an overall period of 4 hr., at a concentration of at least four times that of the zinc in the medium, it is quite probable that under the present experimental conditions an adequate concentration of the respective metal ions would have been present to compete effectively with ^{65}Zn. The uptake values in the presence of the cations, as shown by Table 5, reflect the capacity therefore of the several cations to compete with ^{65}Zn for binding by the erythrocytes. Neither cobalt, which also forms strong complexes with histidine (Hearon, Schade, Levy & Burk, 1947), nor cadmium, which is known to behave very much like zinc in binding to proteins (cf. Tanford, 1952), has any effect on zinc uptake at mM concentration. Fe^{3+} is the most powerful inhibitor of zinc uptake, a very significant finding in view of the fact that several investigators have found that one of the biochemical lesions of zinc toxicity is an interference with iron utilization as reflected in abnormally low levels of cytochrome oxidase and catalase (van Reen, 1953; van Reen & Pearson, 1953; Sivarama Sastry & Sarma, 1958).

Probably, in many cells, as in erythrocytes, iron and zinc may bind at similar sites. Holmberg & Laurell (1947) have found that iron can even displace zinc bound by serum albumin. However, the fact that none of the ions is inhibitory with haemolysate (even iron is much less inhibitory than with whole erythrocytes) reveals that the influence of other ions is mostly exerted at the cell surface.

However, unlike the penetration of ^{59}Fe into erythrocytes (Boiron *et al.* 1955) and the influx of Mn^{2+} into ascites tumour cells (Pal, 1958) the influx of ^{65}Zn is inhibited by the amino acids. It is not surprising that histidine, an amino acid whose capacity for binding zinc is capable of quantitatively accounting for zinc binding by proteins (Tanford, 1952; Gurd & Goodman, 1952), is also the strongest inhibitor of ^{65}Zn uptake by the erythrocytes. The influence of the amino acids, as shown by Table 6, reflects only their effect on the transport of ^{65}Zn across the erythrocyte membrane since they have no effect on ^{65}Zn uptake by the haemolysate.

SUMMARY

1. It has been shown that equine erythrocytes take up ^{65}Zn from iso-osmotic sodium chloride solution and bind it strongly. The bound zinc was stable to dialysis and was associated to a major extent with haemoglobin. The uptake of ^{65}Zn by whole erythrocytes as well as by a haemolysate prepared from them was studied under different conditions and it was shown that the total zinc bound by the whole cells was somewhat less than the total binding capacity of the erythrocyte proteins for zinc.

2. Very little of ^{65}Zn was bound at the cell membrane, most of it diffusing across the cell wall and becoming bound to intracellular erythrocyte proteins. This uptake process was rapid in the first 2 hr. and was thereafter slow, reaching a maximum in 4 hr.

3. Ethylenediaminetetra-acetate completely inhibited uptake of ^{65}Zn by whole erythrocytes but detached bound ^{65}Zn only very slowly. Among several metabolic inhibitors tested, cyanide markedly increased the uptake of zinc by erythrocytes.

4. Fe^{3+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Cd^{2+} and Cu^{2+} ions were tested for their capacity to influence uptake of ^{65}Zn by erythrocytes. Only Fe^{3+} was powerfully inhibitory at mM concentration; it was much less inhibitory towards uptake by haemolysed erythrocytes.

5. Histidine, lysine, glutamic acid, leucine, glycine, arginine and methionine inhibited uptake of ^{65}Zn by erythrocytes to varying degrees. At 1 mM, histidine was the most inhibitory, depressing uptake by 90%. However, none of the amino acids had any effect of the uptake by erythrocyte haemolysate.

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The Mechanism of the Formation of Higher Alcohols from Amino Acids by *Saccharomyces cerevisiae*

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Recent studies (SentheShanmuganathan, 1956; SentheShanmuganathan & Elsden, 1958) of the formation of tyrosol, one of the constituents of fusel oil, have established that in *Saccharomyces cerevisiae* the conversion of tyrosine into tyrosol is effected by the following reactions.

Tyrosine + 2-oxoglutarate →
p-Hydroxyphenylpyruvate + glutamic acid (1)

p-Hydroxyphenylpyruvate →
p-Hydroxyphenylacetaldehyde + CO_2 (2)

p-Hydroxyphenylacetaldehyde + reduced DPN →
p-Hydroxyphenylethanol (tyrosol)
+ oxidized DPN (3)

In reaction (1), the removal of the amino group is effected by transamination; 2-oxoglutarate was found to be essential for this step. This is followed by decarboxylation of the keto acid (reaction 2) to yield the aldehyde, which is reduced in the third stage to give the alcohol.

In the first investigation, tyrosine was chosen as a substrate because of the relative ease with which the intermediates and the product could be estimated (SentheShanmuganathan & Elsden, 1958). Many other amino acids give rise to fusel oils, and, having established the pathway of tyrosol formation, it was desirable to extend the work in order to decide whether other fusel oils were formed by a similar mechanism. This paper describes these investigations.

In experiments with growing cultures and whole cells, where glucose is an essential component, ethanol was always formed in addition to the alcohol corresponding to the amino acid added as substrate. The presence of ethanol made the estimation of other alcohols difficult. The cell-free extracts used would not reduce added aldehyde in the absence of a hydrogen donor and the amino acid is only converted as far as the corresponding aldehyde containing one less carbon atom than the parent compound. Moreover, there is no formation of ethanol by unrelated reactions or by endogenous metabolism. Consequently, the work reported in this paper has been done with cell-free extracts.

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Since the first reaction in the formation of fusel oil was found to be catalysed by transaminase it was thought desirable to study some of the properties of the transaminase with leucine as the substrate. Although transamination reactions have been studied extensively with preparations from animal tissues (Braunstein & Kritzman, 1937; Feldman & Gunsalus, 1950; Cammarata & Cohen, 1950; Rowsell, 1956*a*, *b*; Canellakis & Cohen, 1956), very little work has been done with yeast. Thus transamination reactions have been demonstrated only in three species of yeasts: *Torulopsis utilis* (Roine, 1947), *Saccharomyces fragilis* (Bigger-Gehring, 1955) and brewer's yeast (Holzer, Gerlach, Jacobi & Gnoth, 1958).

MATERIALS

Organism. The organism used throughout this work was *Saccharomyces cerevisiae* (strain 5, isolated in this Department). The method of growing the cells and the preparation of cell-free extracts have been described previously (SentheShanmuganathan & Elsden, 1958).

Amino acids. All the amino acids used (L. Light and Co. Ltd., Colnbrook, Bucks) were checked for purity by paper chromatography (Woiwod, 1949). Solutions of the amino acids were adjusted to pH 6·2 or 7·2 with 0·2 N-NaOH before use.

2-Oxo acids. The 2-oxo derivatives of caproic acid, *iso*-caproic acid, valeric acid and *iso*valeric acid were prepared from their corresponding DL-amino acids by the D-amino acid oxidase method of Meister (1952). 2-Oxoglutarate was prepared by the method of Clutterbuck (1927). Oxaloacetate was a gift from Professor Sir Hans Krebs, F.R.S.; the sodium salt was prepared from it as described by Elsden & Ormerod (1956). Phenylpyruvate was obtained from L. Light and Co. Ltd. *p*-Hydroxyphenylpyruvate was prepared as described earlier (SentheShanmuganathan & Elsden, 1958). Indolylpyruvic acid was prepared from indole. Indole-3-aldehyde was synthesized as described by Boyd & Robson (1935). The azlactone was prepared from the aldehyde by condensing it with hippuric acid (Ellinger & Matsuoka, 1920) and the product hydrolysed with NaOH. The hydrolysate was poured into ten times its volume of ice-cold water and the solid precipitate filtered off. The supernatant was acidified and extracted with ether. After drying with anhydrous Na_2SO_4, the ether was evaporated under reduced pressure at room temperature, leaving a brownish white solid. The indolylpyruvic acid was separated from benzoic acid by extraction of the latter with light petroleum

(b.p. 60–80°). The crude product was purified and crystallized as described by Woods (1935).

Aldehydes. Butyric, *iso*butyric, valeric and *iso*valeric aldehydes (L. Light and Co. Ltd.) were distilled just before use. Aqueous solutions of the aldehydes were standardized by the $NaHSO_3$-binding method.

Alcohols. Pentan-1-ol, 3-methylbutan-1-ol, butanol and 2-methylpropan-1-ol (L. Light and Co. Ltd.) were distilled just before use.

Other compounds. Hexose diphosphate was prepared as described by SentheShanmuganathan & Elsden (1958). Diphosphopyridine nucleotide (DPN) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.) and crystalline yeast alcohol dehydrogenase from Boehringer und Söhne Mannheim, Germany. Reduced DPN was prepared from DPN by the alcohol dehydrogenase method of Pullmann, Colowick & Kaplan (1952). Pyridoxal phosphate was the gift of Dr A. L. Morrison of Roche Products Ltd., Welwyn Garden City, Herts.

METHODS

Protein. Protein contents of preparations were determined by the turbidimetric method of Stadtman, Novelli & Lipmann (1951). It was assumed that a solution containing 0·1 mg. of protein/ml. gave an extinction of 0·1 at 340 mμ in the Unicam SP. 500 spectrophotometer.

Glutamic acid. The incubation mixtures were deproteinized and centrifuged. The supernatant was brought to pH 4·5 with 2N-acetic acid and the glutamate content estimated by the decarboxylase method of Gale (1945) with Warburg cups of approx. 10 ml. capacity.

2-Oxo acids. 2-Oxoglutarate was estimated by the transaminase method of Krebs (1950). Aliphatic 2-oxo acids were determined by yeast carboxylase (Meister, 1952) and *p*-hydroxyphenylpyruvate by the colorimetric method of Briggs (1922) as modified by Canellakis & Cohen (1956).

Qualitative test for glutamate and other amino acids. For this purpose the incubation mixtures were inactivated by heating in a boiling-water bath for 10 min. The denatured proteins were centrifuged off and equal volumes of the supernatant placed on paper chromatograms. The procedure of Woiwod (1949) was adopted for the separation of the amino acids. After the papers had been dried, the positions of the amino acids were located by spraying with 0·1% (w/v) ninhydrin in acetone (Dent, 1947).

Preparation of extracts for assay of transaminase activity. Washed whole cells were prepared (SentheShanmuganathan & Elsden, 1958) and crushed in the bacterial press (Hughes, 1951). The crushed mass was extracted with 12 ml. of 0·1 M-phosphate buffer (0·072 M-Na_2HPO_4–0·028 M-KH_2PO_4), pH 7·2/g. dry wt. of cells (dry weight was determined turbidimetrically by using a Hilger Spekker absorptiometer with a neutral-grey Ilford filter no. H. 508) for 1 hr. at 37° and centrifuging at 19 600 *g* for 20 min. at −2°. The insoluble material (P) was resuspended in an equal volume of the same buffer for testing transaminase activity. The supernatants (pH 6·5–6·7) were brought to pH 7·2 with 0·2N-NaOH and dialysed against 0·02 M-KCl at 1–2° for 16–18 hr. with continuous stirring to remove glutamate and other amino acids. The dialysed extract and the precipitate were tested for transaminase activity between 2-oxoglutarate and tyrosine or leucine; the precipitate was inactive. Such a dialysed extract contained 28 mg. of protein/ml.

Assay of transaminase activity. The formation of glutamate from 2-oxoglutarate and the appropriate amino acid was used as an index of transaminase activity.

Unless otherwise stated, incubations were in duplicate at 25° in test tubes which contained, in a total volume of 3 ml.: pyridoxal phosphate (0·04 μmole), enzyme preparation, 2-oxoglutarate, amino acid and 2 ml. of phosphate buffer, pH 7·2 [see Tables and Figures for concentrations]. The mixture was incubated for 10 min. at 25° and the reaction then started by addition of the amino acid (Canellakis & Cohen, 1956). Two controls, one without amino acid and the other without 2-oxoglutarate, were included in all experiments; no detectable glutamic acid was formed in either of the controls.

At the end of the incubation period 0·2 ml. of 50% trichloroacetic acid was added, the tubes were plunged into a boiling-water bath for 3 min. and the denatured proteins separated by centrifuging.

Manometry. When gas exchanges were measured, reaction mixtures were incubated in Warburg manometers with double side bulbs by conventional techniques. Reactions were initiated by the addition of the cell-free extracts from the side bulb. Unless stated otherwise, all manometric experiments were carried out at 25° in phosphate buffer, pH 6·2, in air. The reaction was stopped by the addition of 0·5 ml. of 10% trichloroacetic acid from the second side bulb. This served to liberate the bound CO_2, to inactivate the enzymes and to precipitate the proteins. The mixture was then centrifuged and the supernatant used for analysis.

RESULTS

When leucine, norleucine, valine or norvaline and 2-oxoglutarate were incubated with cell-free extracts reinforced with pyridoxal phosphate, CO_2

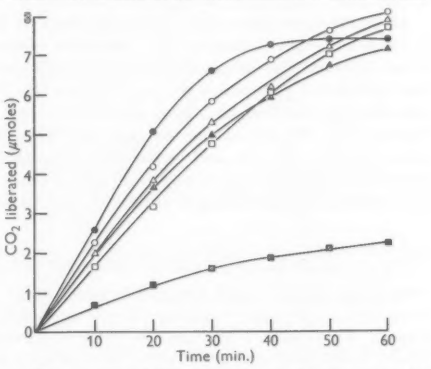

Fig. 1. Time course of CO_2 liberation in the system amino acid plus 2-oxoglutarate by cell-free extracts. Experiments were carried out in Warburg flasks with double side bulbs. The main compartment contained L-amino acid (10 μmoles), 2-oxoglutarate (100 μmoles), pyridoxal phosphate (0·01 μmole) and water to 1·7 ml. Reaction was initiated by the addition of extract (1 ml.) from one of the side bulbs. Gas phase, air; temp. 25°. ○, Valine; △, norvaline; □, norleucine; ●, *iso*leucine; ▲, leucine; ■, no amino acid added.

was liberated in excess of the amount formed in the absence of the amino acid (Fig. 1). When pyridoxal phosphate was omitted, the rate of CO_2 liberation decreased. At the pH used (6·2) there is some retention of CO_2, and the total CO_2 produced was obtained by the addition of acid at the end of the experiment. Since CO_2 retention was small, the rate of gas formation was used as a rough index of the progress of the reaction. 2-Oxoglutarate could not be replaced by either pyruvate or oxaloacetate.

Products of the reaction. The production of CO_2 which occurred when amino acids were incubated with the extract, 2-oxoglutarate and pyridoxal phosphate suggested that transamination followed by decarboxylation of the resulting 2-oxo acid to yield the aldehyde was occurring. This was tested by analysing the reaction mixture for glutamate and aldehyde at the end of the experiment. An approximate estimate of the amount of aldehyde was obtained by steam-distillation into thiosemicarbazide, and the thiosemicarbazenes formed were determined by their absorption at 260 mμ. Although recoveries with pure aldehydes or with aldehydes produced from 2-oxo acids by the action of purified yeast carboxylase were quantitative, recoveries of aldehydes added to reaction mixtures were only in the order of 80 % (Table 1). The CO_2 production in the absence of added amino acid was probably due to the slow decarboxylation of 2-oxoglutarate by the cell-free extract. Table 2 shows that the CO_2 produced, corrected for that of the control, was equivalent to the glutamate formed. The recoveries of the aldehydes were, as expected, low.

In order to confirm that aldehydes were in fact produced in the reaction, the aldehyde formed when leucine was incubated with 2-oxoglutarate and pyridoxal phosphate was isolated as the 2:4-dinitrophenylhydrazone. The procedure was as follows. To 100 ml. of the extract in 0·1 M-phosphate buffer, pH 6·2, and containing 0·5 μmole of pyridoxal phosphate, was added 50 ml. of the substrate mixture containing 2 m-moles of DL-leucine and 10 m-moles of 2-oxoglutarate (pH of the substrate mixtures was preadjusted to 6·2 with dilute NaOH soln.). A sample (3 ml.) of the incubation mixture was transferred to a Warburg manometer. The incubation was carried out at 25° for 5 hr., by which time gas output in the manometer had ceased.

At the end of the incubation period the pH of the mixture was adjusted to 1·0 with 5 N-HCl and the precipitated proteins were separated by centrifuging at 0° for 10 min. at 19 600 *g*. The supernatant was adjusted to pH 9·0 with dilute sodium hydroxide soln. to prevent distillation of succinic semialdehyde, and steam-distilled. The distillate (50 ml.) was collected in a saturated solution of 2:4-dinitrophenylhydrazine (50 ml.) in 2 N-HCl, the receiver being kept in an ice bath. The mixture was left at 0° overnight. The derivative was purified as described earlier for *p*-hydroxyphenylacetaldehyde (SentheShanmuganathan & Elsden, 1958). The melting point of the compound was 122–124° and the mixed melting point with an authentic sample of *iso*valeraldehyde 2:4-dinitrophenylhydrazone (m.p. 122–123°) was 121–123° (Found: C, 49·5; H, 5·56; N, 21·12. $C_{11}H_{14}O_4N_4$ requires C, 49·6; H, 5·26; N, 21·05 %).

The control experiment which contained no DL-leucine gave no precipitate with 2:4-dinitrophenylhydrazine under these conditions.

Tests with 2-oxo acids. If transamination is the first step in the conversion of the amino acids into their corresponding alcohols, the intermediates will be the corresponding 2-oxo acids and aldehydes containing one less carbon atom than the parent amino acid. 2-Oxocaproic acid, 2-oxo*iso*caproic acid, 2-oxovaleric acid and 2-oxo*iso*valeric acid, which would be expected to arise from norleucine, leucine, norvaline and valine respectively, were tested and all were found to give CO_2 and aldehyde with extracts at pH 6·2 and at 25° (Table 3).

Purified carboxylase prepared from *S. cerevisiae* (strain 5) by the method of Green, Herbert & Subrahmanyan (1941) also decarboxylated these

Table 1. *Effect of interfering substances present in the experimental system on the recovery of butyraldehyde*

The experimental system contained, in a total volume of 10 ml.: 3·59 μmoles of butyraldehyde, 2ml. of extract or 160 μmoles of 2-oxoglutarate or both at pH 6·2. Incubated at 25°; gas phase, air. Samples were withdrawn at intervals of 30 min., deproteinized with trichloroacetic acid and stored at 0° until required. After adjustment to pH 9·0 with 0·1 N-NaOH the aldehyde was steam-distilled in a Markham apparatus. The distillate (50 ml.) was collected in a tube containing 10 μmoles of thiosemicarbazide in 2 ml. of 0·5 M-acetate buffer, pH 4·0. After standing for 30 min. at 37° the thiosemicarbazones were determined by their absorption at 260 mμ.

	Extract		2-Oxoglutarate		Extract + 2-oxoglutarate	
Time (min.)	Aldehyde found (μmoles)	Recovery (%)	Aldehyde found (μmoles)	Recovery (%)	Aldehyde found (μmoles)	Recovery (%)
0	2·3	64	3·59	100	3·18	88
30	2·3	64	3·00	83	3·12	87
60	1·9	52	2·64	73	2·37	66

four 2-oxo acids almost quantitatively (Table 4) to yield the aldehyde and CO_2. The aldehydes produced in these experiments were also estimated by the thiosemicarbazone method. The carboxylase of the strain of *S. cerevisiae* used in these experiments had a specific activity of 6·1 units/mg. of protein [1 unit: that amount of enzyme required to catalyse the liberation of 100 μl. of CO_2 in 3 min. under the conditions specified by Green *et al.* (1941)]. *p*-Hydroxyphenylpyruvic acid was not decarboxylated by the purified enzyme when 0·694 mg. of protein (4·2 units of activity) was present. However, when 4 mg. of protein (24·4 units of activity) was used, *p*-hydroxyphenylpyruvic acid was

Table 2. *Products of the reaction from amino acids plus 2-oxoglutarate by undialysed cell-free extract*

All experiments were carried out in Warburg vessels with double side bulbs. The main compartment contained L-amino acid (10 μmoles), 2-oxoglutarate (100 μmoles), pyridoxal phosphate (0·01 μmole). One of the side bulbs contained extract (1 ml.) in 0·1 M-phosphate buffer, pH 6·2; the second side bulb contained 0·5 ml. of 10% trichloroacetic acid. Total vol., 3 ml. Incubation period, 60 min. Gas phase, air; temp. 25°.

Amino acid	Initial rate of CO_2 liberation (μmoles/10 min.)	Total CO_2 liberated (μmoles)	Glutamic acid formed (μmoles)	Aldehyde recovered (μmoles)
None	0·75	2·4	2·1	0·9
Leucine	2·00	12·6	12·0	6·3
Isoleucine	2·55	10·6	9·9	5·5
Norleucine	1·75	13·4	12·9	7·5
Valine	2·25	11·3	10·5	5·0
Norvaline	2·00	9·7	9·5	4·3
Alanine	0·75	2·4	2·1	—

Table 3. *Decarboxylation of 2-oxo acids by undialysed cell-free extracts of* Saccharomyces cerevisiae *and products of the decarboxylation*

All incubations were carried out in Warburg vessels with double side bulbs until CO_2 evolution ceased. The main vessel contained the 2-oxo acid at pH 6·2, one of the side bulbs contained 1 ml. of extract in 0·1 M-phosphate buffer, pH 6·2, the other contained 0·5 ml. of 10% trichloroacetic acid. Total volume, 3 ml. Temp. 25°; gas phase, air; incubation period, 30 min.

2-Oxo acid	Substrate added (μmoles)	Initial rate of CO_2 liberated (μmoles/3 min.)	Total CO_2 liberated (μmoles)	Recovery of aldehyde (μmoles)
None	—	0·30	0·73	0·90
Pyruvic acid	6·5	3·8	6·72	4·9
2-Oxovaleric acid	11·65	5·8	11·70	6·1
2-Oxo*iso*valeric acid	12·0	3·8	7·94	5·0
2-Oxocaproic acid	12·5	5·8	12·24	6·3
2-Oxo*iso*caproic acid	13·65	3·8	11·60	7·8
p-Hydroxyphenylpyruvic acid*	10·0	0·31	5·23	—

* The incubations were carried out for 60 min.

Table 4. *Decarboxylation of 2-oxo acids by purified carboxylase and estimation of aldehydes by the thiosemicarbazone method*

Incubations were carried out in Warburg vessels with double side bulbs. The purified carboxylase [6·1 units of activity/mg., cf. Green, Herbert & Subrahmanyan (1941)] equivalent to 0·694 mg. of protein, was used for the test from Expts. 1–7. The 2-oxo acid was kept in the main vessel in citrate buffer, pH 6·0, and the carboxylase was in the side bulb in the same buffer, pH 6·0. Gas phase, air; temp. 25°; incubation period, 30 min.

Expt. no.	2-Oxo acid	Substrate added (μmoles)	Initial rate of CO_2 liberated (μmoles/3 min.)	Total CO_2 liberated (μmoles)	Aldehyde recovered (μmoles)
1	None	—	0	0	0·9
2	Pyruvic acid	16·0	5·6	16·2	—
3	2-Oxovaleric acid	12·5	4·1	12·4	11·9
4	2-Oxo*iso*valeric acid	12·5	2·5	11·8	11·0
5	2-Oxocaproic acid	15·0	2·8	13·4	13·0
6	2-Oxo*iso*caproic acid	12·5	2·0	11·4	11·4
7	*p*-Hydroxyphenylpyruvic acid	10·0	0	0	—
8	*p*-Hydroxyphenylpyruvate*	10·0	1·2	4·8	—

* In Expt. no. 8, the amount of carboxylase used was equivalent to 4 mg. of protein; incubation period, 60 min.

decarboxylated (Table 4) slowly. The purified carboxylase obtained by Green *et al.* (1941) and the cell-free preparation of the yeast obtained by Meister (1952) also did not catalyse the decarboxylation of this keto acid.

Reduction of the aldehydes by reduced diphosphopyridine nucleotide. The oxidation of reduced DPN by dialysed extract in the presence of butyric, *iso*butyric, valeric and *iso*valeric aldehydes at pH 8·8 was investigated spectrophotometrically. The reduced pyridine nucleotide was oxidized in all cases but the rate of oxidation decreased with increasing length of the carbon chain of the aldehyde (Fig. 2). Similar observations were made with *p*-hydroxyphenylacetaldehyde (SentheShanmuganathan & Elsden, 1958). The reverse reaction, reduction of DPN in the presence of the corresponding alcohols, was also demonstrated.

The oxidation of reduced DPN by the aldehydes in the presence of crystalline yeast alcohol dehydrogenase was also investigated (Fig. 3). It was found that the rate of oxidation of reduced DPN (as measured by the change in extinction at 340 mμ/min.) by butyric aldehyde was about seven, four and two and a half times that of *iso*butyraldehyde, *iso*valeraldehyde and valeraldehyde respectively. *p*-Hydroxyphenylacetaldehyde was also found to be capable of oxidizing reduced DPN in the presence of the crystalline enzyme.

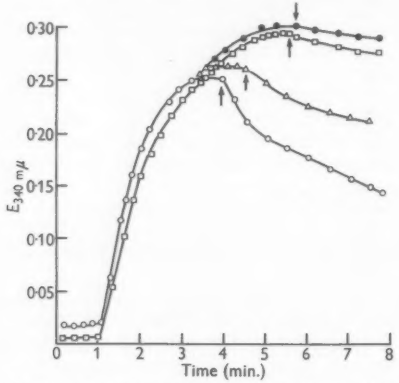

Fig. 2. Oxidation of reduced DPN by aliphatic aldehydes by crude cell-free extracts of *S. cerevisiae*. The reaction mixture (3 ml.) contained pyrophosphate buffer, pH 8·8 (30 μmoles), cobalt sulphate (1 μmole), hexose diphosphate (10 μmoles), DPN (0·3 mg.) and 0·5 ml. of extract. When DPN was reduced, aldehyde was added as indicated by the arrows. The reference cell contained no DPN. ○, Butyraldehyde (1·35 μmole); △, *iso*butyraldehyde (1·37 μmole); □, valeraldehyde (1·14 μmole); ●, *iso*valeraldehyde (1·33 μmole). The amounts of aldehyde added were estimated by the $NaHSO_3$-binding method.

Properties of leucine-2-oxoglutarate transaminase of Saccharomyces cerevisiae

Effect of pH. The pH optimum was found to be between 7·2 and 7·4 (Fig. 4). There was a sharp drop in activity below pH 7·0 but above this value the effect was relatively small over the range studied. For subsequent experiments, pH 7·2–7·4 was chosen, with a buffer concentration of 0·067 M-phosphate. Bigger-Gehring (1955) found that the pH optimum for the leucine-2-oxoglutarate transaminase of *Saccharomyces fragilis* was 8·0. It is difficult to advance an explanation for such a big variation in the pH optimum, for the two organisms belong to the same genus. The extracts were prepared by the same method (Hughes, 1951), the only difference is that Bigger-Gehring incubated her preparations anaerobically at 37° for 2 hr. whereas in these experiments the incubations were at 25° for 30 min.

Effect of 2-oxoglutarate concentration. The amount of glutamate formed in 30 min. increased with increasing concentration of 2-oxoglutarate up to 2 μmoles/μmole of leucine (Fig. 5). Above this

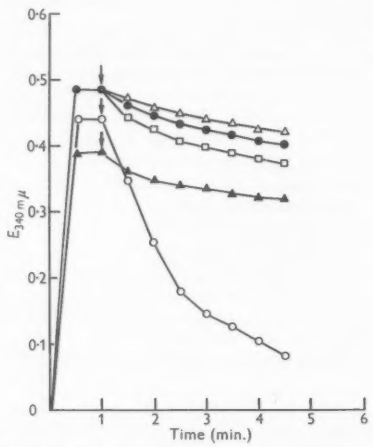

Fig. 3. Oxidation of reduced DPN by aldehydes in the presence of crystalline alcohol dehydrogenase. The reaction mixture (3 ml.) contained pyrophosphate buffer, pH 8·8 (30 μmoles), reduced DPN (0·1 ml. giving an extinction of 0·5 at 340 mμ) and crystalline alcohol dehydrogenase (75 μg.). Aldehydes (0·1 ml.) were added as indicated by the arrows. The reference cell contained no reduced DPN. ○, Butyraldehyde (1·35 μmole); △, *iso*butyraldehyde (1·37 μmole); □, valeraldehyde (1·14 μmole); ●, *iso*valeraldehyde (1·33 μmole); ▲, *p*-hydroxyphenylaldehyde [0·665 μmole estimated as tyrosol (cf. SentheShanmuganathan & Elsden, 1958)]. The amounts of aldehyde added were estimated by the $NaHSO_3$-binding method.

concentration there were signs of inhibition; therefore in all subsequent experiments the amount of 2-oxoglutarate was fixed at 2 μmoles/μmole of amino acid.

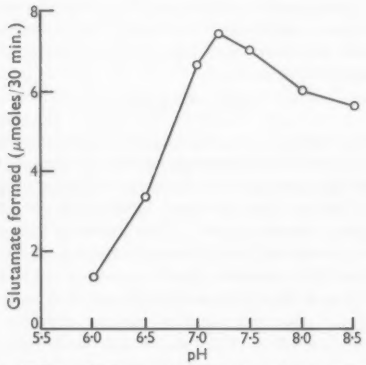

Fig. 4. Effect of pH on the formation of glutamate from leucine plus 2-oxoglutarate by dialysed cell-free extracts of *S. cerevisiae*. Experiments were carried out in test tubes. The complete system (3 ml.) contained pyridoxal phosphate (0·04 μmole), enzyme preparation, 2-oxoglutarate (20 μmoles) and amino acid (10 μmoles) in 0·067 M-phosphate buffer of the desired pH. The reaction was started by the addition of the substrate, amino acid, after first incubating the constituents for 10 min. Two controls, one without the amino acid and the other without 2-oxoglutarate, were included; no glutamic acid was detected in either. The pH remained constant throughout. Temp. 25°, incubation period, 30 min.; gas phase, air.

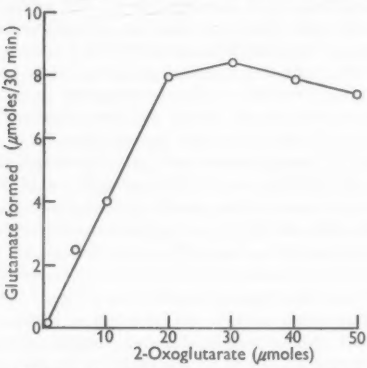

Fig. 5. Effect of 2-oxoglutarate on the formation of glutamate from leucine plus 2-oxoglutarate by dialysed cell-free extracts. Experimental details were as described in Fig. 4. Incubations were made at 25° in 0·067 M-phosphate buffer, pH 7·2. The amounts of 2-oxoglutarate added were varied and the glutamate formed in 30 min. was estimated. No glutamate was formed when the amino acid or 2-oxoglutarate was omitted.

Formation of glutamate with time. The progress of transamination of 2-oxoglutarate with leucine was found to be linear with time over the period 0–40 min. (Fig. 6).

Transamination with other amino acids. Under the optimum conditions obtained for the leucine-2-oxoglutarate system, the transfer of amino groups from a number of amino acids to 2-oxoglutarate was tested with both dialysed and undialysed extract of *S. cerevisiae* after an incubation period of 60 min. Of the 21 amino acids tested, only 15 gave rise to glutamate in the presence of undialysed extract and 10 with the dialysed preparation (Table 5). The activity varied considerably from amino acid to amino acid. With undialysed extract, maximal activity was obtained with aspartic acid, whereas leucine and phenylalanine showed a slightly slower rate; the activities of isoleucine, norleucine, valine, norvaline, methionine and tyrosine were approximately half that of aspartate. Tryptophan and histidine showed the least activity and the other amino acids tested (Table 5) did not transaminate at all.

Effect of pyridoxal phosphate on the formation of glutamate. The results (Table 6) show that pyridoxal phosphate stimulates the synthesis of glutamate and that the degree of activation varies with different amino acids. This was most marked when valine was the substrate. The variable response to the co-factor could mean that more than one transaminase is involved.

Reversibility of the transaminase reactions. A mixture containing the 2-oxo acid (20 μmoles)

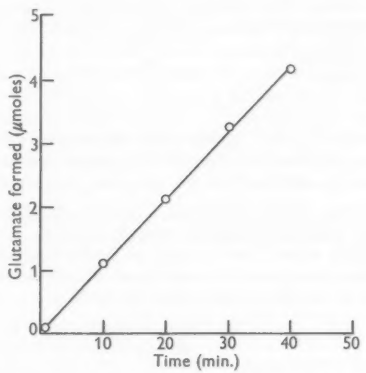

Fig. 6. Progress curve of glutamate formation from leucine plus 2-oxoglutarate by dialysed cell-free extracts. For experimental details see Fig. 4. Incubations were made at 25° in 0·067 M-phosphate buffer, pH 7·2. Glutamate was estimated at 10 min. intervals. No glutamate was produced when the amino acid or 2-oxoglutarate was omitted.

corresponding to the amino acid was incubated with glutamic acid (10 μmoles), pyridoxal phosphate (0·04 μmole) and dialysed extract at 25° for 30 min. in 0·1 M-phosphate buffer, pH 7·2. The control experiment contained no glutamate. At the end of the experimental period the proteins were denatured by heating in a boiling-water bath for 10 min. and separated by centrifuging. Equal volumes of the supernatant were placed on paper chromatograms. The transamination reactions, involving aspartic acid, phenylalanine, tyrosine, tryptophan, leucine, norleucine, valine and norvaline, were reversible. Alanine was not synthesized from pyruvate and glutamate.

Table 5. *Formation of glutamate from 2-oxoglutarate and amino acids by dialysed and undialysed cell-free extracts of* Saccharomyces cerevisiae

Experiments were carried out in test tubes and the system contained, in a total volume of 2·5 ml.: 15 μmoles of the L- or 30 μmoles of the DL-amino acid, 30 μmoles of 2-oxoglutarate, pyridoxal phosphate (0·04 μmole), 1 ml. of 0·1 M-phosphate buffer, pH 7·2, and 1 ml. of extract in 0·1 M-phosphate buffer, pH 7·2. All solutions were adjusted to pH 7·2.

	Glutamate (μmoles/10 mg. of enzyme protein in 60 min.)	
Amino acid	Undialysed preparation	Dialysed extract
None	2·8	0
DL-Aspartic acid	15·3	10·6
DL-Leucine	11·4	8·6
DL-Isoleucine	9·7	6·6
DL-Phenylalanine	10·9	6·8
DL-Valine	10·0	6·8
DL-Norleucine	10·0	6·1
DL-Norvaline	10·5	4·3
DL-Tyrosine	8·8	4·2
DL-Methionine	9·0	3·4
DL-Tryptophan	4·7	0·8
DL-Histidine	4·7	0
DL-Serine	4·2	0
L-Proline	3·9	0
L-Hydroxyproline	3·8	0
DL-Threonine	3·7	0
DL-Alanine	2·8	0
L-Arginine	2·8	0
α-Aminobutyric acid	2·8	0
L-Cystine	2·8	0
L-Ornithine	2·8	0
DL-Lysine	2·8	0

Table 6. *Effect of pyridoxal phosphate on the formation of glutamate from 2-oxoglutarate and amino acids by dialysed cell-free extracts*

The system (2·5 ml.), containing the 2-oxoglutarate (20 μmoles), pyridoxal phosphate (0·04 μmole), 1 ml. of 0·1 M-phosphate buffer, pH 7·2, and 0·5 ml. of extract, was incubated for 10 min. before the substrate (10 μmoles) was added. Temp. 25°; period of incubation, 60 min.

	Glutamate (μmoles/10 mg. of protein enzyme in 60 min.)	
Amino acid	Without coenzyme	With coenzyme
None	0	0
DL-Aspartic acid	5·3	10·6
DL-Leucine	3·4	8·4
DL-Phenylalanine	1·7	6·2
DL-Tyrosine	1·0	3·3
DL-Valine	0·6	5·3

DISCUSSION

Transamination in yeast was first demonstrated by Roine (1947) in *Torulopsis utilis*. He found that a direct transfer of amino groups of alanine, aspartic acid, valine, leucine and isoleucine to 2-oxoglutarate was catalysed by extracts of the yeast. Similar observations were made by Bigger-Gehring (1955) with *S. fragilis*. In the present study it has been found that dialysed extracts of *S. cerevisiae* catalysed the transfer of amino groups from 10 amino acids to 2-oxoglutarate. 2-Oxoglutarate could not be replaced with either pyruvate or oxaloacetate; these findings are in agreement with those of Roine (1947) and Bigger-Gehring (1955). In contrast to the observations of these authors, alanine was not transaminated with 2-oxoglutarate by extracts of the yeast employed under the conditions of the standard test used (i.e. 0·1 M-phosphate buffer, pH 7·2). Bigger-Gehring made all her tests at pH 8·0 and this may explain this difference of activity.

It is seen from Table 2 that the rate of liberation of carbon dioxide from the mixture of amino acid and 2-oxoglutarate by extracts of *S. cerevisiae* is greater than that obtained when the amino acid was omitted. Moreover, the rate of carbon dioxide liberation from the 2-oxo acids (Table 3) corresponding to the amino acids is very much greater than the liberation of carbon dioxide from the amino acid plus 2-oxoglutarate by a similar extract. These results indicate that the carboxylase in yeast is probably responsible for the decarboxylation reaction (2). This was confirmed by the observation that the carboxylase purified from this strain of yeast catalysed the almost quantitative decarboxylation of all the α-oxo acids tested (Table 4).

Of the 2-oxo acids tested *p*-hydroxyphenylpyruvate was least attacked; it is possible that the reaction does not go to completion because the product of the decarboxylation, *p*-hydroxyphenylacetaldehyde, inhibits the enzyme system. This possibility was not investigated any further. Meister (1952) did not observe decarboxylation of *p*-hydroxyphenylpyruvic acid by extracts of yeast, which decarboxylated other 2-oxo acids.

Though *p*-hydroxyphenylpyruvate was not decarboxylated by the same amount of the purified carboxylase as used for the other 2-oxo acids, it was

found that decarboxylation did not take place at a very slow rate if larger amounts of the purified enzyme were used (Table 4). The purified enzyme had 6·1 units of enzyme activity/mg. of protein when assayed under the standard conditions employed by Green *et al.* (1941). In contrast with the present findings, the carboxylase preparation (6·07 units/mg. of protein) obtained by Green *et al.* (1941) from dried brewer's-ale yeast did not catalyse the decarboxylation of this 2-oxo acid. Therefore the present results may indicate the presence of a specific decarboxylase in *S. cerevisiae* (strain 5) for *p*-hydroxyphenylpyruvate and that this is different from the classical pyvuric acid yeast carboxylase.

The third enzyme, which is responsible for reaction (3), is probably yeast alcohol dehydrogenase. The evidence for this hypothesis is supported by the present studies and by the results of Barron & Levine (1952) and Elisuzaki & Barron (1957). Butyric, *iso*butyric, valeric and *iso*valeric aldehydes were tested for their ability to oxidize reduced DPN in the presence of cell-free extracts (Fig. 2). Such studies showed that only butyraldehyde is reduced at an appreciable rate by reduced DPN, whereas the others were reduced very slowly. The reverse reaction, the oxidation of alcohols by DPN in the presence of extracts, was also studied and similar observations were made.

Experiments with crystalline alcohol dehydrogenase confirmed the observations with extracts. Tyrosol was not oxidized by DPN by crystalline alcohol dehydrogenase in the present studies.

The presence of a new alcohol dehydrogenase, alcohol dehydrogenase II, in yeast has been claimed by Elisuzaki & Barron (1957). They have evidence that the new alcohol dehydrogenase did not reduce DPN in the presence of 2-methylpropan-1-ol, but it had higher activity with propanol and butanol than the classical alcohol dehydrogenase. On the other hand, the classical alcohol dehydrogenase showed a slightly higher activity with propan-2-ol, butan-2-ol and ethylene glycol. The oxidation of different alcohols by the intact yeast cells was also studied by Barron & Levin (1952), and they found that the yeast cell could bring about the oxidation of ethanol, propanol, butanol and 2-methylpropan-1-ol. Present studies also showed that the extracts and crystalline alcohol dehydrogenase could oxidize pentyl and *iso*-pentyl alcohols at about the same rate as 2-methylpropan-1-ol. Tyrosol was not oxidized by the crystalline alcohol dehydrogenase in the presence of DPN, and it is not possible to say at present whether the oxidation of tyrosol and the pentyl alcohols by DPN could be effected by alcohol dehydrogenase II. It is therefore suggested from the observations of Barron & Levine (1952), Elisuzaki & Barron (1957) and the writer that the third step in the formation of fusel oil from amino acids is effected by either the classical alcohol dehydrogenase or alcohol dehydrogenase II, or by both. Further studies are required to confirm this hypothesis.

From the previous (SentheShanmuganathan & Elsden, 1958) and present studies it has been concluded that the mechanism of fusel oil formation is of a general nature. The first step in the reaction sequence is a transamination and is most probably catalysed by different transaminases. This is followed by a decarboxylation yielding the aldehyde and carbon dioxide. The reduction of the aldehyde is finally effected by alcohol dehydrogenase via DPN as hydrogen carrier, e.g.

$$\text{Leucine} + \text{2-oxoglutarate} \xrightarrow{\text{transaminase}} \text{2-Oxo}\textit{iso}\text{caproic acid} + \text{glutamic acid} \quad (4)$$

$$\text{2-Oxo}\textit{iso}\text{caproic acid} \xrightarrow{\text{carboxylase}} \textit{iso}\text{Valeraldehyde} + CO_2 \quad (5)$$

$$\textit{iso}\text{Valeraldehyde} + \text{reduced DPN} \xrightarrow{\text{alcohol dehydrogenase}} \textit{iso}\text{Pentyl alcohol} + \text{oxidized DPN} \quad (6)$$

It is relevant to comment on the pH for optimum activity of the various individual enzymes involved in the conversion of tyrosine into tyrosol. The transaminase was found to have an optimum pH of 7·8 (SentheShanmuganathan, 1958), the carboxylase pH 6·0 and alcohol dehydrogenase acts only at a pH above 7·8. In fact all the experiments for alcohol dehydrogenase activity were carried out at pH 8·8. In contrast with these pH optima, the combined reaction transaminase and carboxylase was found to possess an optimum pH of 6·2. It is, however, difficult to advance an explanation of why in experiments with growing cultures and washed cells the conversion of tyrosine into tyrosol could be effected at pH 6·0, a pH at which alcohol dehydrogenase is inactive. This can only be explained on the basis that within the cell the entire system is so orientated that the pH would not be a limiting factor.

One more question remains to be answered, namely, why does yeast form fusel oil? Thorne (1941, 1950) studied the nutrient value of single amino acids and found that though they functioned in the same way of supplying ammonia to yeast they varied considerably among themselves in their nutrient values. Such an assimilation of amino acids probably occurs by the enzymes aspartase (Quastel & Woolf, 1926), glutamic acid dehydrogenase (Euler, Adler & Ericksen, 1937) or glutamic dehydrogenase and transaminase in combination, thus:

$$\text{Glutamic acid} \rightleftharpoons \text{2-Oxoglutarate} + NH_3$$

$$\text{Amino acid}_1 + \text{2-oxoglutarate} \rightleftharpoons \text{2-Oxo acid}_1 + \text{glutamic acid}$$

The availability of nitrogen from an amino acid will, however, depend on the extent to which each amino acid can transaminate with 2-oxoglutarate. From the present studies and those of Bigger-Gehring (1955) there are indications that the ease with which an amino acid can undergo transamination parallels the ease with which the amino acid can be utilized as source of nitrogen, as in the experiments of Thorne (1941).

It seems probable that the real significance of fusel oil formation is that it is a mechanism whereby yeast can utilize amino acids as a source of nitrogen. When simple sources of nitrogen such as ammonium salts and asparagine are available in the medium, fusel oil formation is very much reduced (Ehrlich, 1907), but when amino acids serve as the sole source of nitrogen the production of fusel oil occurs at a very high rate.

SUMMARY

1. Dialysed crude cell-free extracts of *Saccharomyces cerevisiae* catalyse the transfer of amino groups from aspartic acid, leucine, norleucine, isoleucine, valine, norvaline, methionine, phenylalanine, tyrosine and tryptophan to 2-oxoglutarate.

2. The transamination of aspartic acid, phenylalanine, leucine, valine and tyrosine with 2-oxoglutarate is stimulated by the addition of pyridoxal phosphate.

3. When leucine, norleucine, valine or norvaline is incubated with 2-oxoglutarate in the presence of extracts of *S. cerevisiae*, glutamate, carbon dioxide and aldehyde are produced. Addition of pyridoxal phosphate to this system stimulates the rate of formation of carbon dioxide.

4. 2-Oxo*iso*valeric acid, 2-oxovaleric acid, 2-oxo-*iso*caproic acid and 2-oxocaproic acid are rapidly decarboxylated by both cell-free extracts and purified yeast carboxylase to give carbon dioxide and their corresponding aldehydes.

5. *p*-Hydroxyphenylpyruvate is decarboxylated very slowly with large amounts of purified yeast carboxylase.

6. Extracts oxidize reduced diphosphopyridine nucleotide on the addition of *p*-hydroxyphenylacetaldehyde, butyraldehyde, *iso*butyraldehyde, valeraldehyde or *iso*valeraldehyde. Crystalline alcohol dehydrogenase also behaved in a similar manner. The rate of reduction decreased with increasing length of carbon chain.

7. The mechanism proposed for the conversion of tyrosine into tyrosol appears to be a general one for the formation of all the constituents of fusel oils.

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A Study of the Kinetics of the Reaction between Thiol Compounds and Chloroacetamide

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It is commonly assumed that the reaction between sulphydryl groups and alkylating agents proceeds via the RS^- form of the thiol compound. This assumption seems necessary in order to account for the pH-dependence of the reaction, but possibly because of technical difficulties of following the course of the reaction at constant pH, quantitative evidence for this hypothesis is lacking. The development of the pH-stat makes such an investigation possible and the present paper gives an account of the study of the kinetics of reaction of chloroacetamide with mercaptoacetic acid, β-mercaptoethylamine and cysteine. By a study of the pH-dependence of the reaction with the two last-named compounds it was hoped that some light might be shed on their mode of ionization.

EXPERIMENTAL

The mercaptoacetic acid (British Drug Houses Ltd.) was redistilled before use. The mercaptoethylamine hydrochloride was a gift from Evans Chemetics Inc., New York, and the cysteine hydrochloride was prepared by the reduction of cystine with tin and HCl. The chloroacetamide and iodoacetamide were both purified by recrystallization from water before use.

The design of pH-stat used has been described elsewhere (Wood, 1959). Its long-term stability is excellent and for the periods of time occupied by an experiment the drift is completely insignificant. The glass electrode used was a shielded low-resistance type B manufactured by N. L. Jones, Sandringham, Victoria, and no deviation from theoretical slope could be detected between pH 4·0 and 9·0 standard buffers. An external calomel electrode was connected to the reaction vessel by an agar–KCl bridge. The glass reaction vessel of about 30 ml. capacity was double-walled and maintained at constant temperature ($\pm 0{\cdot}02°$) by water circulated through the jacket from a Colora Ultrathermostat (Lorch, Württ, Germany). In addition to the electrodes the reaction vessel also had a stirrer, thermometer and the burette tip from an Agla microsyringe mounted in it. An inlet tube for introducing a stream of O_2-free N_2 into the space over the liquid was also provided. For the kinetic work a direct record of alkali consumption versus time was obtained on a chart recorder (Varian G 10, Palo Alto, Calif., U.S.A. or Honeywell-Brown, 10 mv span, Philadelphia 44, Pa., U.S.A.).

The general technique used was to titrate 10 ml. of the thiol solution (approx. 3 mM) to the required pH in the pH-stat with 0·2 N-KOH in an atmosphere of N_2. Chloroacetamide solution (10 ml.), previously brought to the same temperature (30°), was added and the uptake of alkali followed for a suitable period (10–30 min.). For higher pH values (> pH 9·0) it was also found necessary to adjust the chloroacetamide solution to the required pH before adding it to the thiol solution. Varying the concentration of the chloroacetamide solution provided a convenient means of adjusting the reaction rate to the experimental conditions. Over most of the pH range the reaction could be studied with a ratio of chloroacetamide to thiol in the range 1·5:1 to 8:1. Rate constants were calculated with the integrated form of the equation for a second-order reaction. At low pH values it was found more convenient to use very high ratios of chloroacetamide to thiol and treat the reaction as first order. From the pseudo first-order rate constant the second-order constant can be calculated knowing the chloroacetamide concentration. One of the important estimations from the standpoint of accuracy is the determination of alkali uptake at 100% reaction at the pH value of the experiment. From a knowledge of the pK values of all the groups involved this can be calculated, but would obviously be complex with cysteine or β-mercaptoethylamine since it would involve not only the two pK groups of cysteine but also the pK of the amino group of the reaction product *S*-carbamidomethylcysteine. This complication was avoided by adding iodoacetamide crystals to the reaction mixture when it was decided from the record that the kinetic study had proceeded sufficiently far. Because of the much greater reaction rate of iodoacetamide compared with chloroacetamide, the reaction went to completion in a very short time. Experimental values of the maximum alkali uptake obtained in this way were used throughout the present work. All rate constants were calculated from the data by the least-squares procedure and other derived data used to calculate pK values were similarly treated.

It is not perhaps immediately obvious that the extent of reaction at any time for any given pH is directly proportional to the alkali uptake. This can be demonstrated as follows. Consider a thiol compound, e.g. cysteine or β-mercaptoethylamine, with two dissociation constants K_1 and K_2 in the pH region in which we are interested. Then if we originally have a moles of thiol, the number of moles of alkali required to titrate it from the state where no alkali is bound to a pH corresponding to $[H^+]$ is

$$\frac{aK_1}{K_1+[H^+]}+\frac{aK_2}{K_2+[H^+]}. \qquad (1)$$

If, at any time t, x moles of the thiol have reacted to give a product which now has one dissociation constant K_3, then the mol. of alkali required to maintain the same pH is

$$x+\frac{(a-x)\,K_1}{K_1+[H^+]}+\frac{(a-x)\,K_2}{K_2+[H^+]}+\frac{xK_3}{K_3+[H^+]}. \qquad (2)$$

In this expression the first term represents the acid formed by the reaction, the second and third terms are the alkali bound by the remaining thiol and the fourth term is the alkali bound by the reaction product. The extra alkali (moles) bound as a result of the reaction is thus (2) minus (1)

$$= x\left[1+\frac{K_3}{K_3+[H^+]}-\frac{K_1}{K_1+[H^+]}-\frac{K_2}{K_2+[H^+]}\right]$$
$$= \text{constant} \times x. \qquad (3)$$

The extent of reaction is thus directly proportional to the alkali uptake at any fixed pH, and this is also true for even more complex cases where larger numbers of dissociating groups are involved.

RESULTS

Mercaptoacetic acid

Experiments carried out as indicated above with 10 ml. of 4·027 mM-mercaptoacetic acid and 10 ml. of 7·111 mM-chloroacetamide at 30° gave results which, when plotted for a second-order reaction, gave the family of curves shown in Fig. 1. From these curves apparent second-order velocity constants can be calculated and are tabulated in Table 1.

If the reaction between chloroacetamide and thiol groups proceeds by way of the mercaptide ion, then these rate constants should be proportional to the fraction of the mercaptoacetic acid which is in the form of the RS^- ion. A titration curve of mercaptoacetic acid was carried out in the pH-stat at 30° with a similar concentration of the acid to that used in the kinetic experiments. Readings of alkali consumed were taken at intervals of 0·1 pH unit from pH 9·0 to 10·8, and, after correction for the volume of alkali necessary to bring water to the same pH, these were used to calculate the pK from the Henderson–Hasselbalch equation. The mean value obtained from eighteen readings was 10·24 with a standard deviation of 0·015. An alternative way of handling the data is to plot log $(x/1-x)$ (where x is the molar fraction of alkali consumed) against pH. This gives a straight line giving an intercept on the pH axis where pH = pK. Both techniques gave the same result. Incidentally the acid branch of the titration curve gave an equivalence within 0·1 % of the expected value, assuming 100 % purity. With this value of 10·24 for the pK of the SH group, the molar fraction of the mercaptoacetic acid in the RS^- form can be calculated for the various pH values used in the kinetic work. Fig. 2 shows the result of plotting the apparent rate constant against the fraction of the mercaptoacetic acid in the RS^- form.

Alternatively the results can be used to estimate the dissociation constant of mercaptoacetic acid. Thus the rate of the reaction should be given by the equation

$$\text{rate} = k[RS^-][X], \qquad (4)$$

where k is the true second-order rate constant, $[RS^-]$ is the concentration of mercaptoacetic acid as RS^-, and $[X]$ is the concentration of chloro-

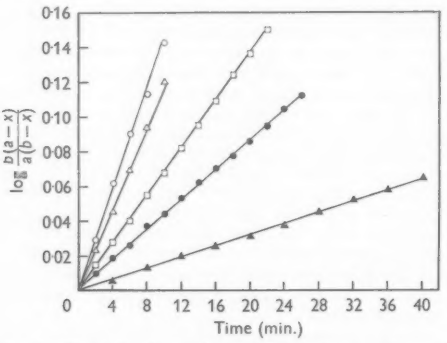


Fig. 1. Second-order rate plots for the reaction of mercaptoacetic acid with chloroacetamide at 30°. ○, pH 10·25; △, pH 10·0; □, pH 9·75; ●, pH 9·50; ▲, pH 9·0.

Table 1. *Variation with* pH *of the apparent second-order rate constant* (k_0) *for the reaction of mercaptoacetic acid with chloroacetamide at* 30°

pH	k_0 (l. moles^{-1} sec.$^{-1}$)
9·00	0·40
9·50	1·09
9·75	1·74
10·00	2·89
10·25	3·70

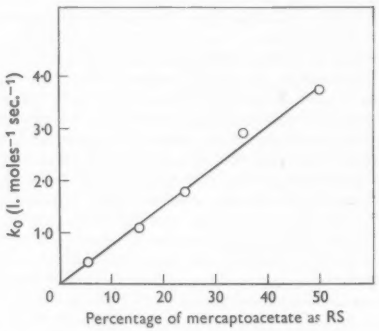


Fig. 2. Apparent second-order rate constants for the reaction between mercaptoacetic acid and chloroacetamide plotted against the molar fraction of the mercaptoacetic acid in the RS^- form.

acetamide. The experimental rate constant (k_0) is defined by the equation

$$\text{rate} = k_0[X]\,([RS^-]+[RSH]), \qquad (5)$$

where $([RS^-]+[RSH])$ is the total concentration of mercaptoacetate.

Equating (4) and (5) we get

$$k_0([RS^-]+[RSH]) = k[RS^-], \qquad (6)$$

therefore $$k_0 = \frac{k[RS^-]}{[RS^-]+[RSH]}.$$

But $$K = \frac{[RS^-]\,[H^+]}{[RSH]}, \qquad (7)$$

where K is the dissociation constant of the mercaptan group and $[H^+]$ is the hydrogen-ion concentration.

Combining (7) and (6) leads to

$$k_0 = \frac{kK}{[H^+]+K}, \qquad (8)$$

or $$\frac{1}{k_0} = \frac{[H^+]}{kK}+\frac{1}{k}. \qquad (9)$$

A graph of $1/k_0$ versus $[H^+]$ should thus be a straight line of slope $1/kK$ and intercept $1/k$. If the experimental data are treated in this fashion we get Fig. 3, which gives values of k 7·68 l. moles^{-1} sec.$^{-1}$ and pK 10·28.

β-Mercaptoethylamine hydrochloride

Amperometric titration of this compound by Dr S. J. Leach suggested that the sample was at least 98 % pure and this was confirmed by titration data. In order to maintain reasonably constant ionic strength the compound (at 3·25 mM) was titrated at 30° in the presence of 0·1 M-KCl with 0·2 N-KOH. The titration curve gave values of 8·11 and 10·48 for the two pK values.

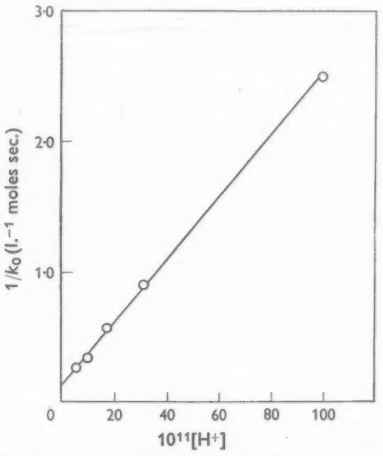


Fig. 3. Plot of the reciprocal of the apparent second-order rate constant ($1/k_0$) against H^+ ion concentration $[H^+]$ for the reaction between mercaptoacetic acid and chloroacetamide at 30°.

Table 2. pH-*Dependence of the apparent second-order rate constant* (k_0) *for the reaction between β-mercaptoethylamine and chloroacetamide at* 30° *and in the presence of* 0·1 M-*potassium chloride*

pH	$10^2\,k_0$ (l. moles^{-1} sec.$^{-1}$)
7·00	0·859
7·25	1·41
7·50	2·44
7·75	3·57
8·00	5·04
8·25	6·76
8·50	8·66
8·75	9·91
9·00	11·0
9·25	11·9
9·50	12·7
9·75	14·5
10·00	16·1
10·25	18·7
10·50	22·1

Table 3. pH-*Dependence of the apparent second-order rate constant* (k_0) *for the reaction between cysteine and chloroacetamide at* 30° *and in the presence of* 0·1 M-*potassium chloride*

pH	$10^2\,k_0$ (l. moles^{-1} sec.$^{-1}$)
6·50	0·239
6·75	0·403
7·00	0·667
7·25	1·152
7·50	1·94
7·75	2·97
8·00	4·24
8·25	5·69
8·50	7·02
8·75	8·72
9·00	9·70
9·25	11·7
9·50	12·9
9·75	14·7
10·00	18·7
10·25	25·0
10·50	40 0

Apparent second-order rate constants for the reaction of the mercaptoethylamine and chloroacetamide in the presence of 0·1 M-KCl were then obtained at intervals of 0·25 pH unit from pH 7·0 to 10·5. The values obtained are listed in Table 2. The interpretation of these results will be discussed jointly with those on cysteine. By the method of Benesch & Benesch (1957) it was shown that reaction of the amino group with chloroacetamide was insignificant under the experimental conditions even at pH 10·5.

Cysteine hydrochloride

Precisely similar data were collected for cysteine. The sample was shown to be 96·5% pure, calculated as cysteine hydrochloride monohydrate. Titration pK values (ignoring the carboxyl group) were 8·10 and 10·36 in the presence of 0·1 M-KCl. Table 3 gives the apparent second-order rate constants for cysteine and chloroacetamide at 30° in the presence of 0·1 M-KCl at intervals of 0·25 pH unit from pH 6·5 to 10·5. Reaction of the amino group of cysteine with chloroacetamide was found to be insignificant.

Interpretation of the results on cysteine and β-mercaptoethylamine

The ionization of cysteine at alkaline pH values was originally postulated as shown in Scheme 1 (see, for example, Cohn & Edsall, 1943).

Calvin (1954) later suggested that the order of ionization of the two groups be inverted, i.e. that the first pK was associated with the ionization of the thiol group, as in Scheme 2.

Edsall (quoted by Ryklan & Schmidt, 1944) postulated that both ionization mechanisms could proceed simultaneously and that the dissociation constants K_1 and K_2 calculated from titration data were composite constants and not directly attributable to any specific group. Edsall's ionization scheme can be formulated as in Scheme 3.

For this complex ionization scheme the following relationships hold between the four dissociation constants K_A, K_B, K_C and K_D, and the two titration constants K_1 and K_2.

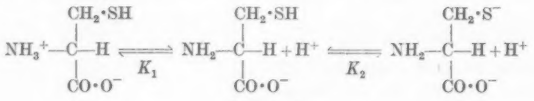
(i) $K_1 = K_A + K_B$,

(ii) $K_1 K_2 = K_A K_C = K_B K_D$,

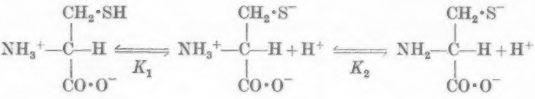
(iii) $\frac{1}{K_2} = \frac{1}{K_C} = \frac{1}{K_D}$.

Cohn & Edsall's formulation (Scheme 1) is a particular case of this general formulation for which $K_1 = K_B$, $K_2 = K_D$ and K_A approaches 0, and Calvin's proposal (Scheme 2) is another special case in which $K_1 = K_A$, $K_2 = K_C$ and K_B approaches 0. Hence a kinetic expression based on Scheme 3 will include (1) and (2) as special cases. With Scheme 3 as the basis for discussing both cysteine and β-mercaptoethylamine and assuming that only RS^- forms react we have

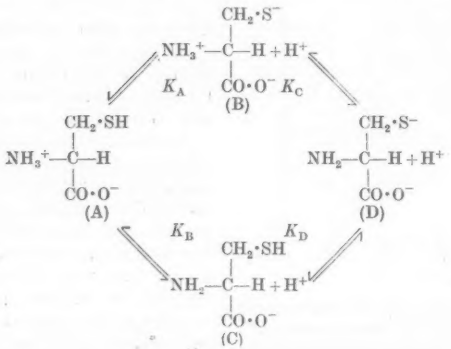
$$\text{rate of reaction} = k_1[B][X] + k_2[D][X], \quad (10)$$



Scheme 1



Scheme 2



Scheme 3

where k_1 and k_2 are the specific rate constants for the two RS^- forms in scheme (3).

Since

$$K_C = \frac{[D][H^+]}{[B]},$$

$$\text{rate of reaction} = \frac{[B][X]}{[H^+]}(k_1[H^+]+k_2K_C). \quad (11)$$

The apparent rate constant k_0 as observable experimentally is defined by the equation

$$\text{rate of reaction} = k_0[X][A+B+C+D]. \quad (12)$$

Equating (11) and (12) and substituting for A, C and D in terms of B, K_A, K_B, K_C and K_D from the various equilibria and making use of relationships (i) and (ii) above leads to the relationship

$$k_0 = \frac{K_Ak_1[H^+]+k_2K_1K_2}{[H^+]^2+K_1[H^+]+K_1K_2}. \quad (13)$$

For Cohn & Edsall's scheme (1), $K_A = 0$, and hence

$$k_0 = \frac{k_2K_1K_2}{[H^+]^2+K_1[H^+]+K_1K_2}. \quad (14)$$

For Calvin's Scheme 2, $K_A = K_1$, and hence

$$k_0 = \frac{K_1[k_1[H^+]+k_2K_2]}{[H^+]^2+K_1[H^+]+K_1K_2}. \quad (15)$$

Equation (13) can be inverted to give

$$\frac{1}{k_0} = \frac{[H^+]^2+K_1[H^+]+K_1K_2}{kK_A[H^+]+k_2K_1K_2}. \quad (16)$$

From the titration data we know that for both cysteine and β-mercaptoethylamine pK_1 and pK_2 are well separated. Hence at pH values < 8·5, K_1K_2 can be neglected in comparison with $[H^+]^2$ and $K_1[H^+]$. Also, unless $k_2 \gg k_1$ and $K_A \ll K_1$, $k_2K_1K_2$ can be neglected in comparison with $k_1K_A[H^+]$. At low pH values equation (16) therefore approximates to

$$\frac{1}{k_0} = \frac{[H^+]}{k_1K_A}+\frac{K_1}{k_1K_A}. \quad (17)$$

A plot of $1/k_0$ versus $[H^+]$ should thus be a straight line of slope $1/k_1K_A$ and intercept K_1/k_1K_A, whence it is possible to determine k_1K_A and K_1. No estimate of k_1 can thus be obtained in the absence of data for K_A. Calvin's formulation (Scheme 2) is such a case since $K_A = K_1$. However, in the absence of any data on K_A only the composite constant k_1K_A can be obtained from kinetic data.

For Cohn & Edsall's formulation (Scheme 1) inversion of (14) gives

$$\frac{1}{k_0} = \frac{[H^+]^2}{k_2K_1K_2}+\frac{[H^+]}{k_2K_2}+\frac{1}{k_2}$$

$$= \frac{1}{k_2}\left[\frac{[H^+]^2}{K_1K_2}+\frac{[H^+]}{K_2}+1\right]. \quad (18)$$

Hence for this case at low pH values a plot of $1/k_0$ versus $[H^+]$ would not be linear, and would become linear only at higher pH values where $[H^+]/K_1 \ll 1$, so that $[H^+]^2/K_1K_2$ is negligible compared with $[H^+]/K_1$.

Figs. 4 and 5 show the result of plotting $1/k_0$ against $[H^+]$ for low pH values for β-mercaptoethylamine and cysteine respectively with the data of Tables 2 and 3. Both sets of data yield straight lines and hence we can conclude that Scheme (1) of Cohn & Edsall cannot be used to interpret the present results on cysteine or β-mercaptoethylamine. The graph also permits us to obtain estimates of K_1 for both compounds directly from the kinetic data. The values obtained in this way

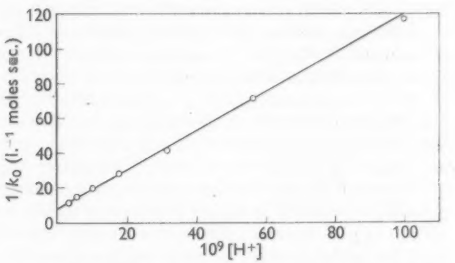


Fig. 4. Plot of the reciprocal of the apparent rate constant ($1/k_0$) against H^+ ion concentration $[H^+]$ for the reaction of chloroacetamide with β-mercaptoethylamine (30°, 0·1 M-KCl; pH values below 8·5).

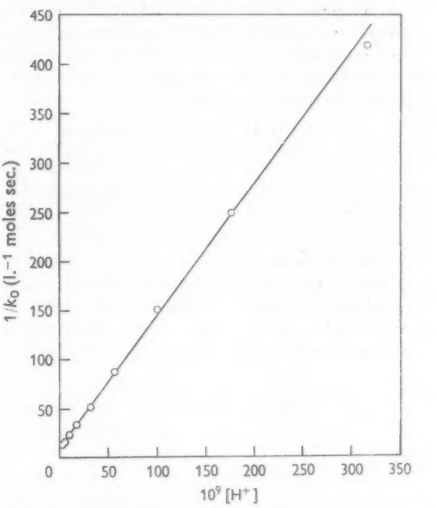


Fig. 5. Plot of the reciprocal of the apparent rate constant ($1/k$) against H^+ ion concentration $[H^+]$ for the reaction of chloroacetamide with cysteine (30°, 0·1 M-KCl; pH values below 8·5).

expressed as pK values are 8·11 for cysteine and 8·11 for β-mercaptoethylamine, in excellent agreement with the values obtained by titration. If Calvin's view is correct and $K_1 = K_A$ then values of k_1 0·119 l. moles^{-1} sec.$^{-1}$ and 0·0959 l. moles^{-1} sec.$^{-1}$ are obtained for β-mercaptoethylamine and cysteine respectively.

Although the data do not enable us to differentiate between ionization Schemes 2 and 3 we can fix limits to the value of K_A with some degree of confidence. The reactivity of the nucleophilic RS^- ion towards reagents such as chloroacetamide will depend in part on the electron density near the sulphur atom. The simultaneous presence of a NH_3^+ group in the molecule will have some tendency to withdraw electrons from the sulphur atom. Thus it seems probable that a compound of this type will react more slowly with chloroacetamide when it is in the form containing a NH_3^+ group than when the amino group is present in the uncharged NH_2 form. Hence we can predict that $k_2 > k_1$ and the condition $k_1 = k_2$ would be limiting. Applying this relationship allows us to predict maximum values for pK_A. Since $K_1 = K_A$ corresponds to minimum values of pK_A we can place rather broad limits on pK_A for cysteine and β-mercaptoethylamine if we know k_2.

The calculation of k_2 and K_2 from the data presents practical problems. If the kinetic studies could be extended to sufficiently high pH values, k_0 would gradually approach k_2 and an estimate could be made on this basis. Alternatively, at very high pH values where $[H^+]^2$ could be neglected in comparison with $K_1[H^+]$, and K_1K_2 and $k_1K_A[H^+]$ can be neglected in comparison with $k_2K_1K_2$, equation (16) can be written as

$$\frac{1}{k_0} = \frac{K_1[H^+]+K_1K_2}{k_2K_1K_2} = \frac{[H^+]}{k_2K_2}+\frac{1}{k_2}. \qquad (19)$$

However, it is not possible experimentally to extend the study to pH values much greater than 10·5 because the amount of acid liberated in the reaction tends to decrease very markedly, and the buffering power of the system becomes considerable so that determination of alkali uptake is subject to considerable error. The only straightforward method of calculating both k_2 and K_2 is thus to substitute k_0, k_1K_A, K_1 and $[H^+]$ in equation (13) and use pairs of values to solve for k_2 and K_2. The difficulty with this procedure is that it is unsystematic and difficult to handle statistically. Alternatively, in the present case we can use the experimental value of K_2 from titration data and solve directly for k_2. As k_2 is the value which has most significance in the present work the latter procedure was adopted. Table 4 summarizes all data obtained on β-mercaptoethylamine and cysteine in this way.

As will be mentioned in the Discussion there have been various estimates of pK_A for cysteine in the literature. Values reported for pK_A (not corrected for ionic strength) are 8·66 (Ryklan & Schmidt, 1944), 8·65 (Grafius & Neilands, 1955) and 8·53 (Benesch & Benesch, 1955), whereas Gorin (1956) quotes data which, interpreted with the corrected value of 8·26 for pK_1 found in the present investigation, lead to an upper limit of 8·72 for pK_A at zero ionic strength.

DISCUSSION

There have been a number of studies which have attempted to determine the sequence of ionizations of cysteine. The earlier attempts (Ryklan & Schmidt, 1944; Grafius & Neilands, 1955) made use of model compounds related to cysteine in which either the SH or NH_3^+ group was modified by alkylation so as to prevent its ionization. From Wegscheider's principle of equivalence the pK of the remaining group is assumed to be unaffected, and this value was used to calculate the remaining dissociation constants in Edsall's formulation. This is obviously only an approximation.

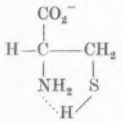
More recently the problem has been tackled by making use of the ultraviolet-absorption band of the RS^- ion (de Deken, Broekhuysen, Béchet & Mortier (1956), Benesch & Benesch (1955) and Gorin (1956)). It is perhaps significant that although all three groups of workers agree closely on their data, they differ markedly in their interpretations. Thus de Deken *et al.* stress the shift in wavelength of the absorption maximum with pH, which they interpret as indicative of hydrogen bonding. In view of this and other evidence they

Table 4. *Summary of data obtained with β-mercaptoethylamine and cysteine at* 30°, *and in the presence of* 0·1 M-*potassium chloride*

	k_1K_A (l. mol.$^{-1}$ sec.$^{-1}$)	k_2 (l. mol.$^{-1}$ sec.$^{-1}$)	pK_A* (maximum value)	pK_1* By titration	pK_1* By kinetic data	pK_2* (by titration)
β-Mercaptoethylamine	$0·922 \times 10^{-9}$	0·299	8·67	8·27	8·27	10·64
Cysteine	$0·762 \times 10^{-9}$	0·429	8·91	8·26	8·27	10·49

* These values have been approximately corrected to zero ionic strength by the factor $0·505\sqrt{I}$.

reject all the three ionization schemes discussed earlier and propose that the only intermediate ionic species can be represented as (I).



(I)

On this view the titration constants are also intrinsic dissociation constants.

Benesch & Benesch, on the other hand, interpret their results in terms of Edsall's scheme and whilst mentioning the shift in absorption maximum with pH make no attempt to interpret it. Their mathematical formulation can be interpreted as identical with equation (13) of the present paper with extinction coefficients replacing rate constants. Although they do not express it in this way, ultimately they arrive at precisely the same situation as has arisen in the present work: they can determine the extinction coefficient of the $R\begin{smallmatrix}S^-\\NH_2\end{smallmatrix}$ form, but the only other parameter directly calculable from their data is a product of K_A and an extinction coefficient. Benesch & Benesch resolve this situation by assuming that the two extinction coefficients are equal and quote evidence to support this assumption. However, as has since been pointed out by Edsall, Martin & Hollingworth (1958), this assumption is not valid though it may be nearly correct. Benesch & Benesch's results could thus be interpreted equally well on Calvin's ionization scheme by assuming an appropriate value for the extinction coefficient of the $R\begin{smallmatrix}S^-\\NH_3^+\end{smallmatrix}$ species. A strict interpretation of their results is thus that they have set limits to the value of K_A. [Although Benesch & Benesch solve their results independently for K_A, K_B and K_D, only one of these can be considered truly independent because of interrelationships (i), (ii) and (iii) above.]

This was realized by Gorin (1956), who took the view that it is impossible to draw any definite conclusions from the study of variation of ultraviolet spectra with pH because the extinction coefficient of the intermediate ionic species is not accessible to experimental measurement.

The failure of all methods so far employed to give an unequivocal answer to the problem of the precise pattern of ionization of cysteine raises the question whether any solution is possible. Looked at from this standpoint it appears that the failure of all methods so far is due to the fact that none of them gives a measure solely of either of the intermediate ionic species. Both the ultraviolet-absorption work and the present kinetic data are basically ways of measuring RS^- but cannot differentiate between

$R\begin{smallmatrix}S^-\\NH_3^+\end{smallmatrix}$ and $R\begin{smallmatrix}S^-\\NH_2\end{smallmatrix}$.

Until the variation in concentration of a single one of these two ionic species with pH can be followed any conclusions drawn must be based on arbitrary assumptions.

For most purposes this conclusion is not quite so unfortunate as might appear at first sight. The work of Benesch & Benesch shows that equilibrium processes for cysteine, as for other polyacids (Adams, 1916), can be interpreted mathematically merely on the basis of titration constants, and the present work shows that this is true also for kinetic data.

Although it is possible to describe satisfactorily the behaviour of cysteine in terms of titration constants for many situations, nevertheless there are problems which cannot be solved without a knowledge of the intrinsic constants. Thus there would be little point in extending the present work to differing temperatures since both k_1 and pK_A could be temperature-dependent and cannot be separately determined. Moreover a knowledge of intrinsic pK values for groups in proteins would enable the distances apart of charged groups to be estimated.

The problem thus has a definite significance but it should be realized that at present no completely unequivocal solution is possible. Fundamentally all that this or previous work has established are limits for the value of pK_A. If analogous work could be done making use of the amino group, limits could be similarly set for pK_B. Combined with the relationship (i) $K_1 = K_A + K_B$ this would permit much closer limits to be set for K_A and K_B. With regard to the present technique any extension of this kind is limited by lack of a suitable reaction which is specific for $R{\cdot}NH_3^+$ or $R{\cdot}NH_2$ in the presence of thiol groups, and spectroscopy of some kind seems the most hopeful possibility.

SUMMARY

1. A study of the kinetics of the reaction between mercaptoacetic acid and chloroacetamide shows that reaction proceeds via the RS^- form.

2. A similar study for β-mercaptoethylamine and cysteine shows conclusively that, when the reactivity of sulphydryl group is considered, the assignment of pK values given by Cohn & Edsall (1943) is incorrect.

3. The kinetic data for β-mercaptoethylamine and cysteine do not permit differentiation between

the ionization schemes proposed by Calvin and Edsall.

4. Kinetic data permit accurate estimates of titration pK values to be made.

5. The problem of the determination of intrinsic dissociation constants is discussed with reference to cysteine.

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Studies on the Particulate Components of Rat Mammary Gland

5. COMPARISON OF LARGE PARTICLES FROM LIVER AND MAMMARY GLAND*

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It is generally considered that the mitochondrial fraction of tissue suspensions contains the major part of the oxidative-enzyme systems of the disrupted cells. In liver tissue the mitochondrial fraction can be separated by differential centrifuging in 0·25 M-sucrose for approximately 100 000g-min. Similar fractions can be obtained, under more or less identical conditions, from a variety of other tissues. This paper emphasizes that large variations can occur between certain tissues in the sedimentation properties of the particles containing such oxidative enzymes as succinic dehydrogenase and cytochrome oxidase, and that the results obtained for sedimentation patterns in liver tissue are not always applicable to other tissues such as mammary gland.

Comparative studies on the mitochondrial fraction from various tissues, contrasting sedimentation properties with enzymic distributions, have seldom if ever been made. Cytological investigations (Weatherford, 1929; Dempsey, Bunting & Wislocki, 1947) and biochemical studies (Greenbaum & Slater, 1957*c*) on rat mammary gland have indicated a change in size of mitochondrial particles over the lactation cycle. Other reports (Dmochowski & Strickland, 1953; Tuba, Orr & Wiberg, 1955) have suggested a size difference between the mitochondria from liver and mammary gland. It was therefore of interest to centrifuge liver and mammary-gland suspensions under identical conditions, tissues from animals at various stages of the lactation cycle being used, to check for: (*a*) possible variations in the size of mammary-gland mitochondria over the lactation cycle; (*b*) possible variations in the size of liver mitochondria over the lactation cycle; (*c*) possible differences between liver and mammary-gland mitochondria.

* Part 4: Slater & Planterose (1958).

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METHODS

The animals used were hooded Norwegian adult female rats (body wt. 180–200 g.) of the Medical Research Council strain. The litters of lactating animals were restricted to 6–11 young. Three stages of the lactation cycle were studied: late pregnancy (18–19 days of pregnancy), 3-days lactating and 18-days lactating. Animals were killed by cervical dislocation and the livers and mammary glands were quickly removed and placed in ice-cold aqueous 0·25 M-sucrose. Livers and mammary glands were homogenized for the same time by the method described by Greenbaum & Slater (1957*a*); after straining through muslin the suspensions were briefly rehomogenized with a plastic pestle (diam. difference, 0·005 in.). Tissue suspensions so prepared were finally diluted (1:5) with ice-cold aqueous 0·25 M-sucrose.

The tissue suspensions of liver and mammary gland from the same animal were centrifuged simultaneously in a MSE Angle 13 centrifuge by the procedure shown in Fig. 1.

Table 1. *Head-radii (r), $g_{max.}$ values and $g_{max.} \times min.$ for the centrifuging steps shown in Fig. 1*

See de Duve & Berthet (1953).

Fraction	$r_{min.}$ (cm.)	r_{max} (cm.)	$10^{-3} g_{max.}$	$10^{-4} g_{max.} \times$ min.
M1	7·7	10·1	4·25	5·15
M2	7·2	10·1	7·25	8·85
M3	6·7	10·1	11·75	13·95
M4	6·1	10·1	16·75	20·35
M5	6·1	10·1	19·65	33·90

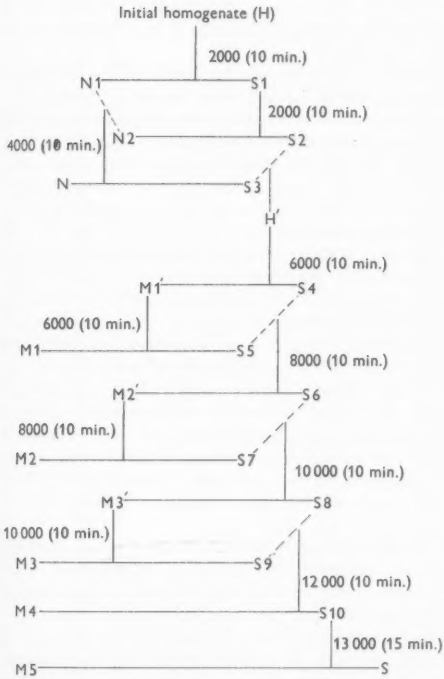

Fig. 1. Centrifuging scheme used for isolation of fractions H′, M1–M5 and S from rat-liver and mammary-gland suspensions. For details see Methods. In the diagram, centrifugings are represented by vertical lines against which are placed the corresponding rev./min. and times used for each stage. The sediment and supernatant suspension resulting from each centrifuging are shown on the left and right respectively of the vertical line. Combination of two supernatant suspensions before recentrifuging is shown by a broken line (- - - -). All homogenate values referred to in the text are relative to that of H′, which is taken as 100.

All operations were carried out at 0°; sediment and supernatant suspension were separated by suction pipettes with bent ends. The constants for each stage of the differential centrifuging are given in Table 1 (cf. de Duve & Berthet, 1953).

Each fraction was finally resuspended in 5 ml. of ice-cold 0·25 M-sucrose. For all assays fraction H′ (Fig. 1) was used as the whole-homogenate standard (i.e. values for H′ are taken as 100%); it can be seen from Fig. 1 that the nuclear fraction and cell debris have been removed from the original tissue suspension in the preparation of H′. Deoxyribonucleic acid phosphorus (DNA-P) measurements on H and H′ showed that only some 10% of the DNA-P in the original suspension (H) came through the centrifugings at 2000 rev./min.

Total protein in the seven fractions H′, M1–M5 and S was determined by the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951). The mean recovery in M1–M5 and S was 105% of H′; results are expressed in terms of the 'recovered' protein and not in terms of the value obtained for H′. Lactose and DNA-P were determined on H′ as reported previously (Greenbaum & Slater, 1957*b*).

Cytochrome oxidase was assayed by a modification of the method of Straus (1956). *NN′*-Dimethyl-*p*-phenylenediamine oxalate was found to be more convenient than the hydrochloride used by Straus. A solution of the oxalate was obtained by stirring 0·11 g. of the solid with about 50 ml. of water at 50–60°; the resulting pinkish solution was diluted to 100 ml. and kept at 0° until used. It was not necessary to neutralize the oxalate before use (as reported by Straus for the hydrochloride), provided that a strong buffer was used in the incubation mixture; recrystallized 2-amino-2-hydroxymethylpropane-1:3-diol (tris), 0·1 M, pH 7·2, was found suitable. Higher concentrations of tris buffer (pH 7·2) had no influence on the reaction. In agreement with Straus (1956), Na^+ ions were found to be inhibitory in the cytochrome-oxidase reaction. Blanks consisted of enzyme fraction, sodium azide, tris buffer, cytochrome *c* and *NN′*-dimethyl-*p*-phenylenediamine oxalate. Cold ethanol was used in all cases to stop the enzymic oxidation. It was found that sucrose inhibits the enzyme reaction by about 15% of a final concentration of 0·06 M. The same amount of sucrose must therefore be added to all tubes. Since the extinction at 540 mμ decreases relatively quickly with time (about 30% in 30 min.) it is essential to keep to a rigid time-table in stopping the reaction and measuring the extinction at 540 mμ.

The conditions adopted for measuring cytochrome oxidase were: a mixture of 1·0 ml. (or 0·9 ml. for the blanks) of tris (0·1 M, pH 7·2); 0·2 ml. of 0·04% cytochrome *c* (Sigma Chemical Co.; crystalline) and 0·2 ml. of enzyme in 0·25 M-sucrose was pre-incubated for 2 min. at 37°. A portion (0·7 ml.) of 0·11% *NN′*-dimethyl-*p*-phenylenediamine oxalate was added and the reaction was stopped (usually 3–5 min. later) by adding 2 ml. of cold ethanol. The tubes were kept in crushed ice until the extinction was measured. The extinction at 540 mμ was determined in a Unicam SP. 600; the path length was 1 cm. for all measurements

given in this paper. Blanks were obtained by a similar procedure except that 0·1 ml. of 0·1 M-sodium azide was added to the incubation mixture. Duplicate tubes were run in all cases.

Under the above conditions the relationship between the amount of tissue added (0–4 mg. wet wt.) and the extinction at 540 mμ was linear. The amount of tissue added was usually 0–2 mg. wet wt. The extinction at 540 mμ increased linearly with the time of incubation at least up to 5 min.; the extinction value increased with the volume of cytochrome *c* added, reaching a plateau value at 0·2 ml.; there was negligible colour formation in the absence of added cytochrome *c*. Boiling the tissue suspensions completely inhibited the reaction. In Table 2 cytochrome oxidase activity is expressed as the extinction at 540 mμ (E_{540}) obtained after 5 min. incubation per mg. wet wt. of tissue.

Succinate–neotetrazolium reductase was assayed by the method of Slater & Planterose (1960) in the presence of added vitamin K_3 and vitamin C. Aerobic incubation was at 37° and was usually for 10 min., except for the most active liver fractions, when a shorter time was used. At least two different concentrations of tissue were used in the assay procedure to check for linearity of response; an equal number of blanks were also measured with sodium malonate in place of sodium succinate in the incubation mixture [for experimental details see Slater & Planterose (1960)]. The activity of succinate–neotetrazolium reductase is expressed in Table 2 as the extinction produced at 510 mμ per min. per g. wet wt. of tissue, the formazan having been extracted into 4 ml. of ethyl acetate.

Succinic dehydrogenase was assayed by the ferricyanide method described by Bonner (1955), modified by a pre-incubation period before addition of substrate to allow for the osmotic swelling of intracellular particles. Under these conditions the addition of substrate produced a linear decrease in extinction at 400 mμ with time.

RESULTS

In preliminary experiments four mitochondrial fractions were isolated from mammary-gland homogenates and the succinic-dehydrogenase activity was measured. For two animals in late pregnancy the percentage of the total activity found in each fraction and the corresponding *g*-min. used in the isolation of the fraction were as follows: M1, 51 500 *g*-min., 54, 49 %; M2, 88 500 *g*-min., 12, 17 %; M3, 109 000 *g*-min., 6, 10 %; M4, 440 000 *g*-min., 7, 11 %; soluble fraction remaining after sedimentation of M4, 20 and 14 %. On other occasions mammary-gland homogenates were separated into large-particle fraction and soluble fraction by centrifuging at 13 000 rev./min. for 15–30 min. in 0·25 M-sucrose, and the soluble fraction was assayed for succinic dehydrogenase and for cytochrome oxidase. Individual values obtained from animals in late pregnancy (expressed as percentages of the homogenate value) were succinic dehydrogenase 21, 29, 18 % and cytochrome oxidase 14 and 21 %.

Soluble fractions from mammary-gland suspensions obtained by removing the large-particle fraction at 13 000 rev./min. for 20 min. were occasionally centrifuged again in a Spinco model L centrifuge at 40 000 rev./min. for 40 min. The microsomal pellet so obtained was assayed for succinic dehydrogenase, succinate–neotetrazolium reductase and cytochrome oxidase. It was found that approximately 20–30 % of the activity appearing in the soluble fraction before high-speed centrifuging was sedimented by the centrifuging at 40 000 rev./min. (i.e. approximately 20–30 % of the activity in fraction S of Fig. 1 is sedimentable at 3×10^6 *g*-min.).

Table 2 gives the results obtained for the variation in total homogenate activity (succinate–neotetrazolium reductase and cytochrome oxidase) over the lactation cycle for both liver and mammary gland. It can be seen that for liver there is a tendency for succinate–neotetrazolium-reductase activity to increase over the lactation cycle whereas cytochrome-oxidase activity remains constant. In mammary gland, succinate–neotetrazolium-reductase activity increases rapidly over parturition ($P < 0{\cdot}001$) and also during lactation ($P < 0{\cdot}001$). Cytochrome-oxidase activity, on the other hand, is virtually unchanged over parturition but increases by a factor of two in lactation (P 0·02).

Table 2 also gives the total protein contents of liver and mammary-gland homogenates (H′); it can be seen that liver protein/g. wet wt. of tissue is constant over the period studied, whereas mammary-gland protein increases approximately twofold at parturition and a further twofold in lactation.

An interesting feature of Table 2 is the percentage of enzyme remaining in the soluble fraction S. Columns 7 and 10 show that only a small amount of each enzyme is in the soluble fraction of rat liver, whereas in mammary tissue the percentage is extremely high (about 40 %) in pregnancy. This high value in pregnancy decreases throughout lactation to a value similar to that obtained for liver.

Columns 8 and 11 (Table 2) give the recoveries of both enzymes in the six fractions assayed (M1–M5 and S) compared with the value obtained for the whole homogenate (H′). Column 8 for succinate–neotetrazolium reductase shows that the recovery is between 80 and 90 % for both liver and mammary gland. For cytochrome oxidase, however, liver values are between 70 and 80 %, whereas mammary gland values are all greater than 100 %.

Table 3 gives the percentage distribution of both enzymes among the five particulate fractions M1–M5. For both enzymes in liver there is no difference in distribution at the three stages studied. In mammary gland there is a tendency for both enzymes to become more associated with M1 and M2 as lactation proceeds. For instance, with cyto-

Table 2. *Protein content and activities of succinate–neotetrazolium reductase and cytochrome oxidase of rat-liver and -mammary-gland suspensions*

All values given in this table are the means of four animals ± standard error of the mean. For details see Methods section.

Tissue	Stage of lactation	Tissue wt. (g.)	Milk content* (% wet wt. of tissue)	Protein content (mg./g.)	Succinate–neotetrazolium reductase			Cytochrome oxidase		
					Activity†	Supernatant‡ activity	Recovery (%)§	Activity‖	Supernatant‡ activity	Recovery (%)§
Liver	Late pregnancy	9·2±0·2	—	249±13	5·74±1·42	10·3±1·7	87±4	90±11·4	4±0·6	68±1·0
	Early lactation	8·2±0·8	—	226±12	7·28±0·74	7·2±2·0	87±6	104±4·7	4±1·2	73±3·8
	Late lactation	9·1±0·6	—	263±43	9·25±1·30	6·7±1·5	88±3	98±2·8	0	77±3·2
Mammary gland	Late pregnancy	4·9±0·2	—	23±3·7	0·06±0·01	36±3·2	80±4	11±1·3	45±5·6	114±6·6
	Early lactation	5·1±0·4	54	41±3·2	0·27±0·05	23±3·0	86±5	9±1·0	27±2·8	112±1·4
	Late lactation	6·2±0·1	39	84±13	1·50±0·26	8±1·1	82±10	20±3·6	5±3·0	111±6·9

* Calculated from Greenbaum & Slater (1957*a*).
† Units: E_{510}/g. of tissue/min.
‡ Expressed as percentage of the activity recovered in all fractions (M1–M5 and S).
§ Activity recovered in all fractions expressed as percentage of homogenate activity (H′).
‖ Units: E_{540}/mg. of tissue/5 min. incubation.

Table 3. *Percentage distribution of activities of succinate–neotetrazolium reductase and cytochrome oxidase among fractions* M1–M5 *of rat-liver and -mammary-gland suspensions*

Total activity in M1–M5 = 100%. All values are the means of 4 animals ± standard error of the mean. For details see Methods section.

Tissue	Stage of lactation	Succinate–neotetrazolium reductase					Cytochrome oxidase				
		M1	M2	M3	M4	M5	M1	M2	M3	M4	M5
Liver	Late pregnancy	82±4·5	10±3·6	2±0·5	4±0·9	2±0·0	80±1·6	11±1·7	4±0·3	3±0·3	2±0·3
	Early lactation	87±1·7	7±1·1	3±0·6	2±0·4	1±0·0	75±1·4	14±0·8	5±0·5	4±0·7	2±0·0
	Late lactation	82±3·1	10±1·6	3±0·0	3±0·8	2±0·4	75±1·1	16±0·6	4±1·0	3±0·7	2±0·4
Mammary gland	Late pregnancy	50±6·2	25±6·4	13±6·4	8±4·0	4±3·6	46±5·2	22±1·2	11±0·9	11±1·7	9±2·0
	Early lactation	71±3·4	13±2·5	7±1·4	4±0·7	5±2·2	59±6·3	20±2·6	6±1·3	7±2·2	7±2·6
	Late lactation	68±6·9	21±5·5	3±1·0	4±1·8	4±1·2	58±4·1	20±10·9	7±0·9	10±5·5	5±5·9

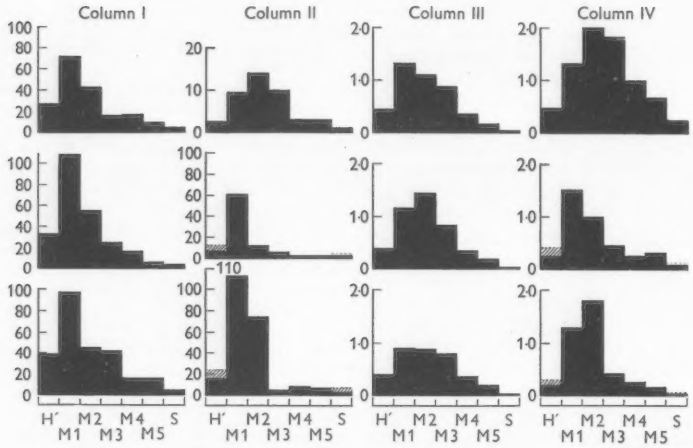

Fig. 2. Distribution of the specific activities of succinate–neotetrazolium reductase and cytochrome oxidase in rat-liver and -mammary-gland suspensions at three stages of the lactation cycle. The three histograms in column I, reading from top to bottom, represent liver succinate-neotetrazolium-reductase specific activity at late pregnancy, early lactation and late lactation respectively; column II, the corresponding values for succinate–neotetrazolium-reductase specific activity for mammary gland; column III, liver cytochrome-oxidase specific activities; column IV, mammary-gland cytochrome-oxidase specific activities. In each histogram the abscissa (reading from left to right) represents the seven fractions, H′, M1–M5 and S. The ordinate in each histogram represents specific activity (i.e. activity/protein content); enzyme activity is defined in Table 2. For mammary gland, the increases in specific activity after correction for retained milk protein are shown for early and late lactation animals by the cross-hatched areas. Each value is the mean of four animals.

chrome oxidase the percentage of the activity in M1–M5 found in fractions M1+M2 in late pregnancy is 68% compared with 78% in late lactation. For succinate–neotetrazolium reductase the corresponding values for M1+M2 are 75% compared with 89%.

Fig. 2 illustrates the variation over the lactation cycle of the specific activities of both enzymes (i.e. enzyme activity/protein content). There is no significant alteration in pattern for liver with either enzyme over the lactation cycle. With mammary gland, on the other hand, there is an accentuation of fractions M1 and M2 for both enzymes as the lactation cycle proceeds. The changes in the specific activities of mammary-gland enzymes are complicated by variations in the amount of milk retained by the gland. It is generally assumed that milk protein appears very largely in the soluble fraction after differential centrifuging so that only the specific activities of the whole homogenate and the soluble fraction would be greatly altered by the presence of retained milk protein. Corrections for retained milk protein made with protein values previously published (Greenbaum & Slater, 1957*a*) and the percentage of milk retained (Table 2, column 4) are shown in Fig. 2 for early and late lactation.

DISCUSSION

Protein content

There is no significant alteration in the amount or distribution of liver protein over the lactation cycle. The changes found for mammary-gland protein are comparable with those previously reported (Slater & Planterose, 1958).

Enzyme activities

There is no change in the cytochrome-oxidase activity of rat liver over the periods studied (Table 2, column 9); there appears to be a tendency for liver succinate–neotetrazolium-reductase activity to increase (Table 2, column 6), although this increase is not statistically significant.

With mammary-gland cytochrome-oxidase activity there is no increase over parturition but an increase occurs over the lactating period from 9 to 20 units (P 0·02). Similar increases in rat-mammary-gland cytochrome-oxidase activity over the lactation period have been reported by Tuba *et al.* (1955) and by Smith & Richterich (1958). Mammary-gland succinate–neotetrazolium-reductase activity increases enormously both over parturition and during lactation; these increases are far greater

than the increases previously reported for succinic oxidase as assayed manometrically (Greenbaum & Slater, 1957*c*; Tuba *et al.* 1955; Smith & Richterich, 1958).

Succinate–neotetrazolium reductase and cytochrome oxidase do not show a similar pattern of increase over the lactation cycle in mammary tissue. In late pregnancy the ratio of homogenate activities (cytochrome oxidase/succinate–neotetrazolium reductase) is 180, whereas in late lactation this ratio is 13. In liver the corresponding ratio is between 10 and 15 for all stages of the lactation cycle.

Enzyme recovery

The recovery of enzyme activity in the six fractions (M1–M5 and S) as a percentage of the activity found in H′ is between 80 and 90 % for the succinate enzyme in both liver and mammary gland (Table 2, column 8). This recovery is similar to the recovery of succinic oxidase from mouse liver (Schneider & Hogeboom, 1950) and the recovery of succinate–cytochrome *c* reductase from rat-liver fractions (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). Considering the relatively large number of centrifugings used (Fig. 1), a recovery of 80–90 % is satisfactory.

The situation is not so clear when the recovery of cytochrome oxidase (Table 2, column 11) is considered. The recovery for liver is lower than might be expected merely from a uniform loss of material during centrifuging. As a result of the complex fractionation used it was not possible to assay either enzyme until about 4 hr. after the start of the experiment. It is therefore conceivable that the isolated fractions deteriorated on standing at 0° more rapidly than the unfractionated homogenate. This seems the probable explanation for the low recoveries found with liver tissue; in several experiments in which only a simple fractionation scheme was used (i.e. separation of one mitochondrial and one microsomal fraction), and for which the assay was performed much sooner after the start of the experiment, the recoveries were all greater than 90 %. A similar explanation accounting for low recoveries of cytochrome oxidase in spleen has been proposed by Eichel (1957).

With mammary gland the apparent recoveries of cytochrome oxidase were consistently greater than 100 % even in experiments where a simple fractionation was used and in which the assay procedure was applied soon after the start of the experiment. Similar high recoveries of cytochrome oxidase have been reported by Brody, Wang & Bain (1952) for brain-tissue fractionations. It seems probable that the high recovery in mammary-gland suspensions is the result of the high fat content of the mammary-gland homogenates; a fat suspension obtained from mammary-gland homogenates has been shown to inhibit the oxidation of *NN′*-dimethyl-*p*-phenylenediamine oxalate in the presence of fresh homogenate, in other words to inhibit the apparent cytochrome-oxidase reaction. In the centrifuging scheme shown in Fig. 1, fat is largely removed during the first high-speed centrifuging at 0°, i.e. in the preparation of M1. Its major effect in the experiments reported in this paper would therefore be on H′, leading to high recoveries. The fact that the recoveries (Table 2, column 11) of cytochrome oxidase in mammary-gland suspensions are all virtually identical at the three stages studied indicates that this fat effect is a constant feature throughout the lactation cycle.

Enzyme distributions

The percentage distributions of both enzymes (Table 3) have been calculated in terms of the total activity recovered and not in terms of the activity of the unfractionated homogenate (see de Duve *et al.* 1955). In this manner comparison between different stages of lactation cycle and between the two tissues can be made with confidence since all assays were performed under identical conditions. Thus the distribution patterns obtained in the above manner for cytochrome oxidase in liver and mammary gland are strictly comparable with the respective distribution patterns for succinate–neotetrazolium reductase, although the percentage recoveries of these two enzymes in terms of the activity of H′ (Table 2, columns 8 and 11) are very different.

The distributions of both succinate–neotetrazolium reductase and cytochrome oxidase in the various intracellular fractions studied in rat liver are comparable with those found in many previous studies on the intracellular distributions of succinic dehydrogenase and cytochrome oxidase. The distributions found for succinate–neotetrazolium reductase and for cytochrome oxidase are indistinguishable from one another at all stages of the lactation cycle. This is also in accord with previous work on succinic dehydrogenase and cytochrome oxidase (see de Duve *et al.* 1955). It is now generally accepted that succinic dehydrogenase and cytochrome oxidase are almost wholly associated with the mitochondrial fraction in rat liver. Table 2 (columns 7 and 10) shows that there is little activity of either enzyme in fraction S. As can be seen from Table 3 (columns 3 and 8), both succinate–neotetrazolium reductase and cytochrome oxidase are largely concentrated in the particulate fraction M1.

In mammary gland, the distributions of both succinate–neotetrazolium reductase and cytochrome oxidase alter in a similar manner over the lactation cycle. In late pregnancy, for both enzymes there is a very high (36–45 %) proportion

of the recovered activity in fraction S (Table 2, columns 7 and 10); this has decreased to half its value by early lactation and is down to a value of less than 10% by late lactation. For each stage of the lactation cycle studied the distribution patterns of succinate–neotetrazolium reductase and cytochrome oxidase are very similar. It can be seen from Table 3 (columns 3 and 8) that fraction M1 in mammary gland does not contain so high a proportion of the total recovered activity as was found for fraction M1 in liver tissue.

The major difference between the enzyme distributions for liver and mammary gland, however, is in the percentage of the recovered activity associated with fraction S. In liver at all stages of the lactation cycle, the activity in fraction S is less than 10%, whereas in mammary gland at late pregnancy the value is around 40%. Since the overall activities of both enzymes in late pregnancy in mammary gland are low the significance of the activity ascribed to fraction S must be discussed with reserve. It will be convenient to consider the two enzymes assayed in turn. (*a*) Cytochrome oxidase. The constitution of the blanks rules out non-enzymic formation of colour as a source of activity in fraction S. Further, it has been shown that the activity apparent in fraction S is proportional to the amount of fraction S added, is dependent on the addition of cytochrome *c* and is destroyed by boiling the tissue suspension. Approximately 30% of the activity in fraction S can be sedimented by centrifuging at 3×10^6 *g*-min. Thus it would seem that the cytochrome-oxidase activity found in fraction S is real. (*b*) Succinate–neotetrazolium reductase. The same arguments can be made in favour of a real activity of succinate–neotetrazolium reductase in fraction S. The activity shown by fraction S is proportional to the amount added, is destroyed by boiling, is dependent on the presence of succinate and is inhibited by malonate (Slater & Planterose, 1960). Additional points can, furthermore, be made. First, the results of Greenbaum & Slater (1957*c*) showed that only some 55% of the total succinic oxidase activity of mammary-gland homogenates was sedimented after centrifuging for approximately 230 000 *g*-min. (approximately equivalent to fraction M5 in this investigation) at late pregnancy, whereas 85% was sedimentable during late lactation. It was in fact suggested that the results inferred a change in size of mammary-gland mitochondria over the lactation cycle (Greenbaum & Slater, 1957*c*); the activity in the supernatant from their experiments would presumably have been approximately 40% in late pregnancy and approximately 10% in late lactation, figures very similar to those obtained in this investigation by a colorimetric as opposed to a manometric method. Secondly, succinic dehydrogenase has been demonstrated in fractions during late pregnancy by the ferricyanide method of Bonner (1955). Thirdly, the distribution of succinic dehydrogenase determined by this method among four mitochondrial fractions and a soluble fraction from mammary glands during late pregnancy was strictly comparable with the results presented in Tables 2 and 3. Thus it would seem that the succinate–neotetrazolium-reductase activity in fraction S is real and that there are indeed large percentages of the total activity of cytochrome oxidase and succinate–neotetrazolium reductase in fraction S in late pregnancy. This activity in fraction S is gradually overshadowed by rapid increases in particulate enzymes as the lactation cycle proceeds.

Tissue differences

Although many reports have been concerned with variations in enzymic composition between mitochondria from different tissues (for instance see Holton, Hülsmann, Myers & Slater, 1957; Aldridge, 1957), no reports have appeared to our knowledge in which the sedimentation properties of mitochondria from liver and another tissue have been directly compared as in this investigation. The results show clearly how large the differences between liver and another tissue can be with respect both to the sedimentation behaviour of particles containing oxidative enzymes and to the composition of the mitochondria themselves. It can be deduced from the results that mammary-gland mitochondria show a more platykurtic distribution than liver mitochondria (i.e. enzyme activity is distributed over a greater range of *g*-min. in mammary gland). These conclusions confirm earlier reports of differences between liver and mammary-gland mitochondria (Dmochowski & Strickland, 1953; Greenbaum & Slater, 1957*c*). If it is assumed that there is no radical alteration in the density of the particles over the lactation cycle then it follows that the sedimentation changes found infer a change in size of the mitochondria in mammary gland over the lactation cycle. This conclusion is in agreement with the cytological results of Weatherford (1929), who reported that mammary-gland mitochondria increase in size from late pregnancy to late lactation.

SUMMARY

1. Liver and mammary-gland homogenates at three stages of lactation have been separated into five large-particle (mitochondrial) fractions. The distributions of succinate–neotetrazolium reductase, cytochrome oxidase and protein among these fractions were studied.

2. In liver there was a tendency for succinate–neotetrazolium reductase to increase from late

pregnancy to late lactation, whereas cytochrome oxidase remained constant. No change in the distribution of these enzymes or of protein was found at the three stages of the lactation cycle studied.

3. In mammary gland succinate–neotetrazolium reductase increased rapidly over parturition and throughout lactation; cytochrome oxidase showed a smaller increase during lactation. These increases were accompanied by changes in the enzyme distribution among the mitochondrial and soluble fractions, more activity being found in the larger particles as lactation proceeds.

4. Mammary-gland mitochondria appear to change in size over the lactation cycle, being smallest in late pregnancy and largest in late lactation. In late lactation they sediment at approximately the same *g*-min. values as do liver mitochondria.

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An Assay Procedure for a Succinate–Neotetrazolium-Reductase System

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There is a rapid production of reduced neotetrazolium chloride (formazan) by rat-liver suspensions in the presence of succinate, vitamin C and vitamin K_3 (Slater, 1959*b*). The enzyme system coupling the reduction of neotetrazolium chloride with the oxidation of succinate is termed succinate–neotetrazolium reductase. The adoption of the word 'reductase' in connexion with neotetrazolium chloride as final electron acceptor must, of course, be interpreted with reserve. It is not intended to imply that there is only one route for the reduction of neotetrazolium by succinate as it seems probable that the pathway operating in the unsupplemented homogenate is different from that which operates in the presence of added vitamin C and vitamin K_3. This paper describes suitable conditions for the assay of such an enzyme system in the presence of added vitamin K_3 and vitamin C; under the conditions described formazan production increases proportionately with the amount of tissue added.

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METHODS

The animals used were hooded Norwegian rats of the Medical Research Council strain. They were adult females, body weight 180–200 g., and were fed on a diet described by Parkes (1946). Liver homogenates were prepared either in water or in 0·25 M-sucrose at 0° in a conventional-type homogenizer with a plastic pestle (diam. difference 0·005 in.). Mammary-gland homogenates were prepared in

the type of homogenizer described by Chaikoff & Emanuel (1957). Vitamin K_3 (2-methyl-1:4-naphthaquinone) was added as a suspension (10 mM unless otherwise stated) as described for vitamin E suspensions by Deul, Slater & Veldstra (1958). The suspensions were freshly prepared on each occasion as follows: 25 mg. of vitamin K_3 was dissolved in 3 ml. of ethanol; this solution was then added to 12 ml. of 0·2% ox-plasma albumin in 0·1 M-sodium phosphate buffer, pH 7·4. The standard buffer mixture used in the incubations was prepared as follows: 20 ml. of sodium phosphate buffer (Na_2HPO_4–NaH_2PO_4), pH 7·4, 0·1 M; 2 ml. of disodium succinate (recrystallized from water), pH 7·4, 0·5 M; 1 ml. of sodium ethylenediaminetetraacetate (EDTA), pH 7·4, 0·1 M. All references to individual components of the standard mixture, i.e. phosphate buffer, imply that the molarity, pH, etc., are the same as given above.

Tissue (0–20 mg. wet wt.) was pre-incubated aerobically at 37° for 2 min. with standard buffer mixture and vitamin C unless it is specifically mentioned that vitamin C was omitted from this pre-incubation mixture. Except where otherwise stated, 1 μmole of vitamin C was added in a volume of 0·1 ml., and buffer mixture to give a pre-incubation volume of 1·1 ml. was added. Blank values to compensate for reduction of neotrazolium by enzymic and non-enzymic pathways other than via succinate oxidation were obtained by substituting disodium malonate (pH 7·4, 0·1 M) for disodium succinate in the standard buffer mixture. After the pre-incubation period, neotetrazolium chloride (0·15 ml. of aqueous 1% solution, unless otherwise stated) and vitamin K_3 (the quantity added is described at the appropriate position in the text) were added. The reaction was stopped 5–10 min. after the addition of neotetrazolium with 1 ml. of 10% trichloroacetic acid; the formazan was extracted by shaking with 4 ml. of ethyl acetate and the extinction was obtained at 510 mμ with a Hilger Uvispek spectrophotometer.

RESULTS

The production of formazan by succinate–neotetrazolium reductase in unsupplemented homogenates does not increase proportionately with the amount of tissue added (Slater, 1959*b*). Formazan production is greatly stimulated by the addition of vitamin K_3 although the response remains non-linear (Slater, 1959*b*). At three different amounts of liver tissue (5, 10 and 15 mg. wet wt.) maximum stimulation of formazan production by vitamin K_3 in the absence of vitamin C was obtained with a final vitamin K_3 concentration of approximately 1 mM. Table 1 gives the results of this investigation, for which the incubation time was 5 min. Higher levels of vitamin K_3 than 1–2 mM depress formazan production by low amounts of tissue possibly because of the inhibitory effect of ethanol on the enzyme system. The addition of 0·1, 0·2 and 0·3 ml. of ethanol to 20 mg. wet wt. of liver tissue in 1 ml. of standard buffer mixture inhibited formazan production by 14, 80 and 94% respectively during a 20 min. incubation. A portion (0·1 ml.) of vitamin K_3 suspension contained only 0·02 ml. of ethanol. Similar results to those reported for rat liver were obtained with rat-mammary-gland suspensions although on occasions a higher level (2 mM) of vitamin K_3 was required for optimum response; this is probably due to the high fat content of rat-mammary-gland homogenates.

Formazan production increases as the concentration of neotetrazolium in the incubation medium is increased. In a typical experiment 5 mg. wet wt. of liver tissue was incubated with 0·7 μmole of vitamin K_3 and varying amounts of neotetrazolium. Buffer mixture to give a final incubation volume of 1·1 ml. was added. Formazan production reached a plateau value when more than 1 mg. of neotetrazolium was added; no inhibition of the reaction was observed when up to 3 mg. of neotetrazolium was added to the incubation medium.

With 1 mM-vitamin K_3 in the incubation medium the effect of varying the final ascorbate concentration was investigated. Fig. 1 shows that there is an increase in formazan production as the final ascorbate concentration is increased to approx. 1–2 mM in rat-liver suspensions (5–15 mg. wet wt.). Varying the pre-incubation period from 0 to 10 min. in the presence of ascorbate was without effect on this ascorbate stimulation. It can be seen from Fig. 1 that the very marked non-linear response

Table 1. *Variation in the amount of formazan produced by the addition of vitamin K_3 to three different amounts of rat-liver tissue*

Incubation was at 37° (5 min.) in standard buffer mixture as described in the Methods section; vitamin K_3 was added as a suspension in ethanol + ox-plasma albumin + phosphate buffer as described in the Methods section. Formazan was extracted with ethyl acetate and measured at 510 mμ.

Amount of tissue added (mg. wet wt.)	Vitamin K_3 added in suspension (μmoles)					
	0·10	0·30	0·50	1·0	2·0	3·0
	Formazan produced with various amounts of vitamin K_3 (μg.)					
5	1	47	83	71	6	6
10	63	208	240	276	268	252
15	118	296	281	380	380	330

Table 2. *Effect of increasing concentration of ascorbic acid on the blank value in the presence of malonate as a percentage of the value obtained in the presence of succinate*

Formazan production was measured with three amounts of rat-liver tissue (5, 10 and 15 mg. wet wt.) at five different amounts of vitamin C (0·3 μmole–10 μmoles); pre-incubation was for 2 min. Incubation was in the presence of 1 mM-vitamin K_3 for 8 min. Other details were as described in the Methods section.

Final ascorbate concn. (mM)	Wt. of liver tissue (mg. wet. wt.) 5	10	15
	Formazan (μg.) produced in the presence of malonate / Formazan (μg.) produced in the presence of succinate $\times 100$		
0·3	9	2	2
0·6	4	2	6
1·0	9	5	10
2·0	24	16	19
10·0	71	54	47

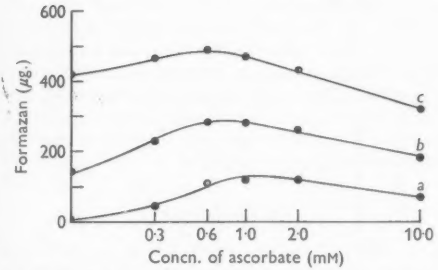

Fig. 1. Formazan production by succinate–neotetrazolium reductase in the presence of varying amounts of ascorbic acid (0–10 μmoles were added) and with three different amounts of rat-liver tissue (curve *a*, 5 mg. wet wt.; curve *b*, 10 mg. wet wt.; curve *c*, 15 mg. wet wt. of liver tissue). Each tube contained 1 mM-vitamin K_3, 0–0·2 ml. of vitamin C solution, 0·15 ml. of neotetrazolium chloride and buffer mixture to give a final incubation volume of 1·35 ml. Pre-incubation was for 2 min. in the presence of buffer mixture, tissue and vitamin C; incubation time after addition of vitamin K_3 and neotetrazolium chloride was 8 min. Other details are as given in the Methods section.

found when no vitamin C was added was largely eliminated when 1–2 mM-vitamin C was present in the incubation medium. Linearity of response in the presence of 2 mM-vitamin C is shown specifically in Fig. 2 for rat-liver and rat-mammary-gland suspensions. If high concentrations of ascorbate are used the succinate–neotetrazolium-reductase system becomes difficult to identify against a very high blank value resulting from the reduction of neotetrazolium by ascorbate and vitamin K_3. Table 2 shows that the blank value (i.e. formazan production in the absence of succinate as substrate) increases rapidly as the ascorbate concentration is increased. At a final ascorbate concentration of 10 mM the blank is 50–70 % of the value obtained in the presence of succinate. With 1 mM-ascorbate,

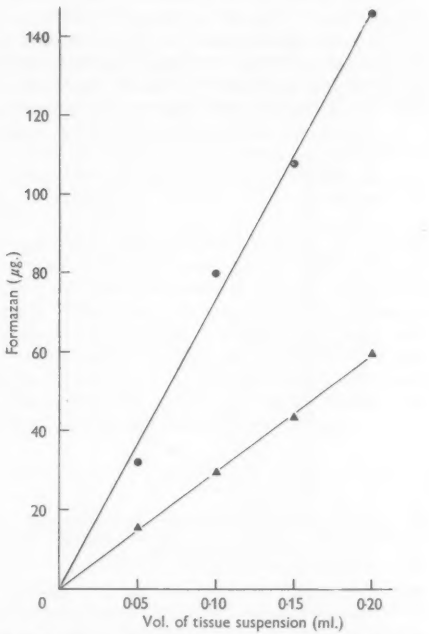

Fig. 2. Formazan production by rat-liver (●) and rat-mammary-gland (▲) suspensions with various amounts of tissue (0–20 mg. wet wt.). Tissue was pre-incubated for 2 min. with 1 ml. of standard buffer mixture, 2·2 μmoles of vitamin C and water to give a final volume of 1·5 ml. Incubation was started by the addition of 0·25 ml. of vitamin K_3–neotetrazolium chloride mixture (0·1 ml. of vitamin K_3 suspension in ethanol + ox-plasma albumin + phosphate buffer–0·15 ml. of 1 % neotetrazolium chloride solution). Incubation time was 8 min. Liver suspension, 1:20 in water; mammary-gland suspension, 1:10 in water, tissue at fourteenth day of lactation; formazan values were corrected for production in the absence of succinate.

however, the blank value is only some 10% of the value obtained in the presence of succinate with 0–15 mg. wet wt. of liver tissue. Formazan production in the presence of malonate appears to be largely the result of a non-enzymic coupling of vitamin C and vitamin K_3 (Slater, 1959*a*); this process is stimulated by tissue suspensions and tissue fractions. Boiling a liver homogenate, however, only decreases this stimulation by some 20%. The stimulatory power of tissue homogenates resides largely in the soluble fraction obtained after removing particulate fractions by differential centrifuging. Examination of the blank values obtained by Slater & Planterose (1960) indicated that the stimulatory power of both rat-liver and -mammary-gland suspensions was distributed as follows among the six fractions they described: M1, 13%; M2–M5, 18%; fraction S, 69%.

Formazan production via the succinate–neotetrazolium-reductase system varies with the time of incubation as shown in Fig. 3. It can be seen that for both liver and mammary-gland suspensions there is a preliminary time lag of approximately 4 min. before formazan production becomes appreciable.

In the presence of 1 mM-vitamin K_3 and 1 mM-vitamin C the assay system is suitable for determining the level of the succinate–neotetrazolium-reductase system in intracellular fractions of rat liver and mammary gland; Fig. 4 illustrates this. It can be seen that under the conditions used formazan production is proportional to the amount of tissue added for all fractions. The overall

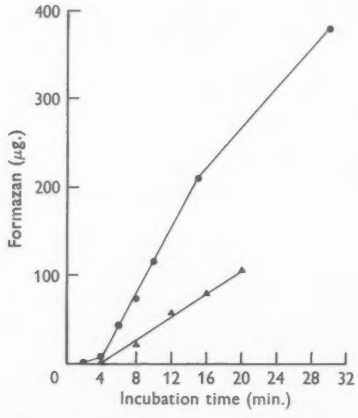

Fig. 3. Formazan production by rat-liver (●) and rat-mammary-gland (▲) suspensions after various times of incubation (0–30 min.). Standard buffer mixture (15 ml.), 3 ml. of 0·2% vitamin C and tissue suspension [2 ml. of liver suspension (1:20); 4 ml. of mammary gland (1:10)] were pre-incubated for 2 min. at 37°. Neotetrazolium chloride–vitamin K_3 mixture (5 ml.: 2 ml. of vitamin K_3 suspension in ethanol + ox-plasma albumin + phosphate buffer–3 ml. of 1% neotetrazolium chloride) was then added to start the incubation. Samples (2 ml.) were withdrawn at various times of incubation and pipetted into 1 ml. of cold 10% trichloroacetic acid. Formazan was extracted and estimated as described in the Methods section. Formazan values are corrected for production in the absence of succinate. The animal used was at the fourteenth day of lactation.

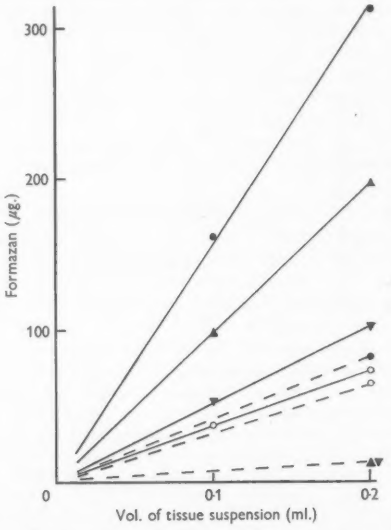

Fig. 4. Formazan production by various intracellular fractions of rat liver. For details of preparation of the fractions see Slater & Planterose (1960). Fraction H′ (●) is the whole-homogenate standard; fractions M1 (▲) and M2 (▼) are mitochondrial fractions; fraction S (○) is the supernatant remaining after the sedimentation of fraction M5 at approx. 340 000 *g*-min. Continuous lines show formazan production in the presence of sodium succinate as substrate; broken lines show the corresponding blank values, i.e. formazan production in the presence of sodium malonate. For clarity, the malonate points obtained with 0·1 ml. of suspension are omitted. The concentrations (fraction H′ in g./ml. of final suspension; other fractions relative to initial concentration in fraction H′) of the fractions used in the assay were: H′, 1:13·4; M1, 1:10·7; M2, 1:2·7; S, 1:1. Each tube contained 88 μmoles of phosphate buffer, 4 μmoles of EDTA, 40 μmoles of sodium succinate or 8 μmoles of sodium malonate, 1 μmole of vitamin C, 1 μmole of vitamin K_3 and 1·5 mg. of neotetrazolium chloride. The final incubation volume was 1·5 ml.; pre-incubation of tissue, buffer and vitamin C was for 2 min.; incubation time was 5 min.; other details were as described in the Methods section.

Table 3. *Formazan production after varying times of incubation at 37° in the absence of added vitamin K_3 and ascorbic acid*

Rat liver or mammary gland (17-days lactating), 20 mg. wet wt., was incubated with 2 ml. of phosphate buffer, 0·2 m of sodium succinate, 0·2 ml. of EDTA and 0·5 ml. of 1% neotetrazolium chloride.

	Incubation time (min.)					
	10	20	30	60	90	120
Tissue	Formazan produced (μg.)					
Liver	36	67	105	209	—	—
Mammary gland	—	—	25	46	65	105

difference between formazan production in the presence of succinate and that in the presence of malonate is small for fraction S; this difference can, however, be obtained with reasonable accuracy by assaying at several levels of tissue.

The formazan solution in ethyl acetate is stable over reasonable periods of time; the extinction at 510 mμ decreases approx. 6% per hr. The succinate–neotetrazolium-reductase system is destroyed by boiling and is inhibited by *p*-chloromercuribenzoate.

DISCUSSION

The short time lag which occurs before a linear production of formazan is obtained is similar to the time lags in tetrazolium reduction mentioned by Shelton & Rice (1957) for succinate oxidation and by Lagnado & Sourkes (1957) for monamine oxidation. This does not appear to be the result of permeability phenomena arising from the relatively large charged molecule of neotetrazolium chloride, since bursting intracellular structures by high-speed blending or by freezing and thawing has little influence on the reaction (Slater, 1959*b*). After this preliminary time lag, however, formazan production is linear until the concentration of formazan becomes limiting. For instance, in the presence of added vitamin K_3 and vitamin C, where the reaction is very fast, linearity is maintained under the conditions used only up to about 15 min. with liver tissue (Fig. 3). In tissue suspensions unsupplemented with vitamin K_3 and ascorbate the reaction is very slow and formazan production via succinate oxidation is linear for 1–2 hr. (Table 3).

The method has been applied to tissues other than rat liver: e.g. rat-kidney, rat-mammary-gland and mouse-liver suspensions. It seems that the concentration of vitamin C required for optimum response varies with the tissue. On a few occasions with rat mammary gland, for instance, a higher concentration of ascorbate was required for linearity of response with increasing amounts of tissue. Higher levels of ascorbate than approximately 2 mM in rat liver depress formazan production by succinate–neotetrazolium reductase (Fig. 1), at the same time greatly increasing formazan production by a process independent of succinate oxidation and probably utilizing a non-enzymic coupling of vitamin K_3 and vitamin C, which are known to interact directly (Slater, 1959*a*). Ascorbic acid has been previously reported to inhibit the succinic-oxidase complex to a larger extent than succinic dehydrogenase itself (Slater, 1949).

A routine assay of a succinate–neotetrazolium-reductase system can be performed under the conditions suggested. Under such conditions the distribution of the succinate–neotetrazolium–reductase system among intracellular fractions of rat liver and rat mammary gland has been examined (Slater & Planterose, 1960). The results showed that the succinate–neotetrazolium-reductase system is almost wholly associated with the mitochondrial fraction in rat liver; the distribution pattern found was identical with that found in many previous studies on the distribution of succinic oxidase in rat-liver suspensions.

SUMMARY

1. A method is described for estimating an enzyme system (succinate–neotetrazolium reductase) coupling the oxidation of succinate with the reduction of neotetrazolium chloride. In the presence of added vitamin K_3 (1 mM) and vitamin C (approximately 1 mM) the production of formazan increases proportionately with the amount of tissue added.

2. Assay conditions are described for liver homogenates applicable to 0–15 mg. wet wt. of liver tissue. The application of the method to intracellular fractions and to other tissues is discussed.

We are grateful to Dr A. L. Greenbaum for many helpful comments and advice. This work was done during the tenure of a Beit Memorial Fellowship by one of us (T. F. S.); the award of a grant from the Agricultural Research Council to D. N. P. is likewise gratefully acknowledged.

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Amino Acid Sequence in Mycobacillin

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It has been observed from the study of physicochemical properties of mycobacillin that it is a polypeptide consisting of seven different amino acids (Majumdar & Bose, 1958). Studies on amino acid composition of mycobacillin show that the molecule is composed of five aspartic acid residues, two glutamic acid and two tyrosine residues and one residue each of serine, alanine, leucine and proline. The problem is how the amino acids are arranged in the mycobacillin molecule. The studies of Sanger and his collaborators (Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953) demonstrated the details of covalent structure of insulin by the application of fairly simple methods of separation and analysis, e.g. paper chromatography, paper electrophoresis and end-group analysis. This paper describes work done to obtain small peptides by a suitable method of hydrolysis followed by fractionation and identification of amino acid residues in each of the peptide fractions, and determination of *N*-terminal amino acids of the constituent peptides to discover the amino acid sequence of the mycobacillin molecule.

EXPERIMENTAL

Partial hydrolysis of mycobacillin. A sample of 30 mg. of mycobacillin was hydrolysed with 3 ml. of 11·4N-HCl for 7 days at 37° in sealed tubes. The hydrolysate was then freed from HCl by evaporating the sample to dryness *in vacuo* below 40° and the process was repeated thrice after addition of fresh water. Finally the hydrolysate was dissolved in 2 ml. of water.

Fractionation of peptide mixtures. After partial hydrolysis the next step was the separation of the complex mixture of peptides. By the application of descending two-dimensional paper chromatography with water-saturated phenol as the first solvent and butanol–acetic acid–water (4:1:1) as second, it was possible to have a satisfactory fractionation of the complex mixture of peptides. About 10 μl. of the sample was spotted at one corner (5 cm. away from both the edges) of the Whatman no. 1 filter paper (56 cm. × 46 cm.). The peptide mixture was allowed to run parallel in six pairs. One of each pair was developed with 0·2% of ninhydrin in acetone and used as a guide to locate peptide areas on the other undeveloped chromatogram. With the aid of the guide chromatogram, individual peptide areas were located, cut out and eluted with water (Consden, Gordon & Martin, 1947). The solution of each eluted peptide was evaporated *in vacuo* to give a residue.

Identification of constituent amino acids of a peptide. In the identification of constituent amino acids, a portion of the peptide residue eluted from the undeveloped chromatograms was completely hydrolysed in 0·5 ml. of 5·7N-HCl at 110° for 18 hr. The hydrolysate was then taken up in water and evaporated under reduced pressure to remove HCl, the operation being repeated twice. The final residue was dissolved in 0·05 ml. of 10% propan-2-ol. The solution so obtained was analysed chromatographically (Sanger & Tuppy, 1951*a*) and the constituent amino acids were identified.

Identification of N-terminal amino acid of a peptide. The *N*-terminal amino acid residue in each peptide from the partial acid hydrolysate of mycobacillin was determined in two ways: (*a*) by deamination with nitrosyl chloride followed by hydrolysis with hydrochloric acid; (*b*) by the dinitrophenyl (DNP) method of Sanger (1945, 1949).

(*a*) Deamination with nitrosyl chloride. In the deamination of each peptide, the method as followed by Consden *et al.* (1947) was used. A portion of the eluted peptide was dissolved in 0·5 ml. of 5·7N-HCl and deaminated with nitrosyl chloride at 30° for 30 min. The deaminated product was dried over solid KOH *in vacuo*, dissolved in water and evaporated *in vacuo* before complete hydrolysis. Finally, identification of amino acids remaining after hydrolysis of deaminated peptides with 5·7N-HCl at 110° for 18 hr. indicated the amino acid destroyed at the *N*-terminal end of the original peptide.

(*b*) Dinitrophenyl method. The DNP method of Sanger was next tried for amino end-group analysis of peptides. In practice, the peptides eluted from four paper chromatograms were dissolved in 1 ml. of water. To the solution was added 100 mg. of $NaHCO_3$ and then a solution of 0·1 ml. of dinitrofluorobenzene in 2 ml. of absolute ethanol (Schroeder & Legette, 1953). The DNP-peptide residue thus formed

was hydrolysed with 0·2 ml. of 5·7 N-HCl at 105° for 16 hr. to give the DNP-amino acid, which was then extracted with peroxide-free ether. DNP-amino acids were next identified by ascending one-dimensional paper chromatography, with (1) the toluene–pyridine–ethylene chlorohydrin–aq. 0·8 N-NH_3 soln. (5:1:3:3) system of Biserte & Osteux (1951) and (2) the *tert.*-amyl alcohol–phthalate system of Blackburn & Lowther (1951). In each paper chromatogram, control samples of DNP-amino acids, synthesized previously by the method of Sanger (1945), were also allowed to run simultaneously.

Identification of N-terminal amino acids in mycobacillin. A separate experiment was carried out to identify *N*-terminal amino acids in mycobacillin by the DNP method of Sanger as described previously, and also by the phenyl-*iso*thiocyanate method of Edman (1950).

Amino acids. For definition of the abbreviations used for amino acids in this paper see *Biochem. J.* (1953), **55**, 5.

Table 1. *Peptides of a partial hydrolysate of mycobacillin*

Peptide spot no. (Fig. 1)	R_F of peptides: In phenol–water	R_F of peptides: In butanol–acetic acid–water (4:1:1)	Amino acids present	Concn.* of amino acids after hydrolysis of peptides: Before deamination	Concn.* of amino acids after hydrolysis of peptides: After deamination	DNP-amino acids identified	Suggested structure†
1	0·18	0·11	Asp	× × × ×	× ×	Asp	Asp.Glu
			Glu	× ×	× ×		
2	0·26	0·12	Asp	×	×	Ser	Ser.Asp
			Ser	×	—		
3	0·26	0·21	Asp	× ×	× ×		
			Glu	× × × ×	×	Glu	Glu.(Asp, Ala)
			Ala	× ×	× ×		
4	0·30	0·15	Asp	×	×	Glu	Glu.Asp
			Glu	×	?		
5	0·35	0·28	Asp	× × ×	×	Asp	Asp.Ala
			Ala	× × ×	× ×		
6	0·36	0·34	Asp	× ×	?	Asp	Asp.Tyr
			Tyr	× ×	×		
7	0·40	0·40	Asp	×	×	Ala	Ala.Asp
			Ala	×	—		
8	0·48	0·30	Asp	× ×	× ×		
			Glu	× ×	× ×	Asp	Asp.(Glu, Tyr)
			Tyr	× ×	× ×		
9	0·50	0·41	Glu	×	?	Glu	Glu.Tyr
			Tyr	×	×		
10	0·52	0·36	Asp	× × ×	× × ×		
			Glu	× × ×	× × ×	Leu	Leu.(Asp, Glu)
			Leu	× × ×	×		
11	0·60	0·42	Asp	× × ×	× ×		
			Glu	× × ×	× ×	Leu	Leu.(Asp, Glu, Ala)
			Ala	× × ×	× ×		
			Leu	× × ×	?		
12	0·74	0·47	Asp	× × ×	×		
			Ser	× × ×	× ×	Asp	Asp.(Ser, Tyr)
			Tyr	× × ×	× ×		
13	0·76	0·56	Asp	× × ×	× ×		
			Glu	× × ×	× ×	Ser	Ser.(Asp, Glu, Leu)
			Ser	× × ×	—		
			Leu	× × ×	× ×		
14	0·77	0·67	Asp	× × ×	× ×		
			Ser	× × ×	—	Ser	Ser.(Asp, Leu)
			Leu	× × ×	× ×		
15	0·80	0·34	Asp	× ×	× ×		
			Ala	× ×	—	Ala	Ala.(Asp, Pro)
			Pro	× ×	× ×		

* The number of crosses indicates the relative intensities of colour given by each amino acid spot with ninhydrin; ?, very weak spot.

† In referring to peptides of known structure, the abbreviations for the residues are joined by a point and where the arrangements of residues are unknown the residues are included in parentheses.

RESULTS

Peptides of a partial hydrolysate of mycobacillin. Results showing the *N*-terminal amino acids of different peptides (Fig. 1) of a partial hydrolysate of mycobacillin are given in Table 1.

It appears from Table 1 and Fig. 1 that of the 15 peptides isolated, there are seven dipeptides, six tripeptides and two tetrapeptides. Identification of *N*-terminal amino acids by the deamination method has been possible for 10 out of the 15 peptides studied, and in peptides 1, 3, 5, 8 and 12 no conclusion can be drawn about the identity of *N*-terminal amino acid because of incomplete deamination. The DNP method, on the other hand, completely establishes the identity of *N*-terminal residues of all the 15 peptides. Both of these methods give identical results in all cases where it has been possible to establish the identity of *N*-terminal amino acids. These findings lead to a possible sequence of amino acids in these peptides as indicated in the last column of Table 1. Aspartic acid and glutamic acid are present in a higher concentration respectively in peptides 1 and 3, although they are not repeated in the sequence, as indicated by their absence in the aqueous layer after ethereal extraction of DNP-peptide hydrolysate. This may be taken to mean that peptides 1 and 3 are not pure and contain free aspartic acid and glutamic acid respectively.

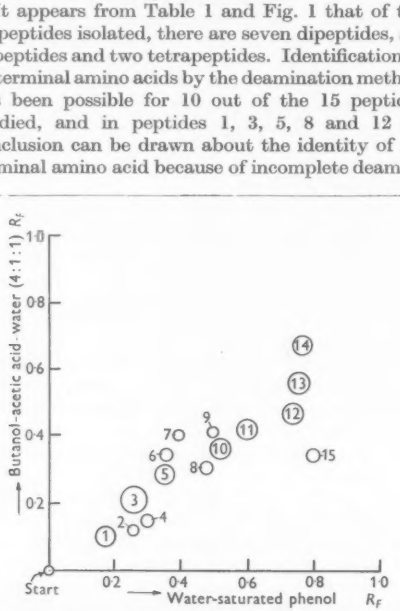


Fig. 1. Two-dimensional chromatograms of peptides in a partial acid hydrolysate of mycobacillin. Intensities of spots with ninhydrin are represented approximately by the areas of the circles.

The knowledge of the amino acid composition and molecular weight (about 1775) of mycobacillin, coupled with the results of determination of *N*-terminal amino acids in each peptide, indicates a possible sequence of amino acids in the molecule as shown in Table 2. It may be noted that mycobacillin contains no free amino group, as shown by the absence of its reaction with ninhydrin and the failure to identify *N*-terminal amino acids in the molecule by the DNP method of Sanger and the phenylthiocarbamyl method of Edman (1950).

DISCUSSION

It will be evident from Table 1 that *N*-terminal amino acids of 15 peptides of a partial acid hydrolysate of mycobacillin are definitely known by the DNP method whereas the method of deamination of peptides followed by hydrolysis has confirmed the *N*-terminal position in 10 out of the 15 peptides.

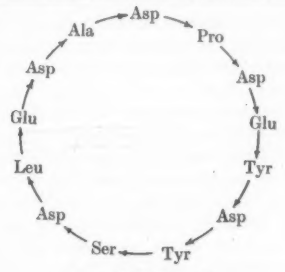
Table 2. *Sequence of amino acids in mycobacillin*

	*1.	Asp.Glu
	8.	Asp.(Glu, Tyr)
	9.	Glu.Tyr
Sequence (I)		Asp.Glu.Tyr
	6.	Asp.Tyr
	12.	Asp.(Tyr, Ser)
	2.	Ser.Asp
	14.	Ser.(Asp, Leu)
	13.	Ser.(Asp, Leu, Glu)
	10.	Leu.(Glu, Asp)
	11.	Leu.(Glu, Asp, Ala)
	3.	Glu.(Asp, Ala)
	4.	Glu.Asp
	5.	Asp.Ala
	7.	Ala.Asp
	15.	Ala.(Asp, Pro)
Sequence (II)		Asp.Tyr.Ser.Asp.Leu.Glu.Asp.Ala.Asp.Pro
		Asp.Glu.Tyr.Asp.Tyr.Ser.Asp.Leu.Glu.Asp.Ala.Asp.Pro†

* Adjacent numbers are peptide spot numbers (Table 1 and Fig. 1).

† The proof of the structure suggested depends on there being two sequences as shown in (I) and (II) above; the relationship between these is not directly proved but, since the peptide is cyclic, they can be joined only in the way shown.

Determination of the chemical structure of tri- and tetra-peptides without the application of the stepwise-degradation method or any method for determining *C*-terminal amino acids has been possible because some of the amino acids such as serine, alanine, leucine and proline are present in the proportion of one molecule each per molecule of mycobacillin. Since these amino acids occur only once in the molecule, the known structure of different dipeptides containing any one of these amino acids has been helpful in determining the possible arrangements of amino acids in tripeptides and tetrapeptides. For example, it will be evident from Table 2 that the dipeptide Ser.Asp gives the possible arrangements of amino acids in the tripeptide Ser.Asp.Leu, and so in the tetrapeptide Ser.Asp.Leu.Glu. Again, the dipeptides Glu.Asp and Asp.Ala show the arrangement of amino acids in the tripeptide Glu.Asp.Ala. Similarly, the structures of other tripeptides and of the tetrapeptide Leu.Glu.Asp.Ala have been established. This knowledge of chemical structure of di-, tri- and tetra-peptides has made it possible to fit together 15 peptide fragments from 13 amino acid residues of mycobacillin molecule into a unique sequence as depicted below. In the absence of a free α-amino group in the molecule. a cyclic structure has been deduced.



The amino acid sequence of antifungal polypeptide antibiotics from *Bacillus subtilis* has not been reported yet, except for bacillomycin, for which only a partial sequence of amino acids has been worked out (Turner, 1956).

SUMMARY

1. Mycobacillin was partially hydrolysed and the hydrolysate fractionated by two-dimensional paper chromatography to give a separation of complex mixtures of peptides.

2. Altogether 15 peptides, including seven dipeptides, six tripeptides and two tetrapeptides, were isolated.

3. The *N*-terminal residue of each peptide was determined by both the deamination method and the dinitrophenylation method.

4. As the mycobacillin molecule does not contain an *N*-terminal residue, a cyclic structure has been suggested.

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The Chemistry of Connective Tissues

5. THE ELASTASE ACTIVITY OF PROTEOLYTIC ENZYMES*

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The dissolution of elastin by pancreatic extract was first demonstrated by Balo & Banga (1950), but since that time there have been many conflicting views about the mechanism of elastolysis. Hall, Reed & Tunbridge (1952) attributed a mucolytic function to pancreatic elastase, Lansing, Rosenthal, Alex & Dempsey (1952) suggested that the action might be lipolytic, Pepler & Brandt (1954) considered that sulphatase action might be involved and Partridge & Davis (1955) concluded that the dissolution of the fibrous protein is brought about by the rupture of peptide links.

Hall *et al.* (1952), from histological and chemical studies, suggested that the fundamental structural element of elastic tissue consists of two chemically distinct components, and that the fibres behaved as though composed of bundles of inner fibrils embedded in a mucoprotein matrix or sheath. During dissolution by pancreatic elastase there was a release of polysaccharide and sulphate, which led to the suggestion that elastase was not a proteolytic enzyme but rather a mucase. Banga & Balo (1953) supported the mucolytic view when they observed the release of reducing substances during elastase action and concluded that elastin was a glucoprotein containing 10–12% of carbohydrate resembling glucosamine, but this figure was subsequently reduced to below 2% (Banga, 1953). Hall (1955) traced the removal of collagen, polysaccharide and sulphate from elastic tissue during the progress of various purification procedures, including the action of hot dilute acetic acid, alkali and urea, and concluded that these methods of freeing so-called elastin from associated proteins are either inadequate or so drastic as to commence the degradation of the fundamental unit of elastic tissue itself. Hall & Gardiner (1955) advanced evidence that pancreatic elastase consists of two enzymes which act on two separate components of elastic-tissue preparations. Banga & Balo (1956), recognizing the heterogeneity of the pancreatic-elastase preparations then in use, suggested the limitation of the name elastase to the enzyme which has proteolytic activity. They reported the presence in purified preparations of pancreatic elastase of an enzyme which they called elastomucoproteinase, which released mucoprotein from elastic tissue. Hall (1957) showed that pancreatic-elastase preparations subjected to electrophoresis revealed the presence of two enzymes, one of which was inactive except in the presence of the other. He suggested that one of these enzymes is involved in the hydrolysis of the mucoprotein sheath and the other in the proteolytic hydrolysis of the central fibril.

Enzymes other than pancreatic elastase have been reported to have elastolytic activity. Thus Yokota (1957) demonstrated that a commercial preparation of α-amylase dissolved elastin, and Pepler & Brandt (1954) claimed that a preparation of bacterial chondrosulphatase was also active. Lansing *et al.* (1952) believed that elastin could be classified as a lipoprotein and observed the release of lipid during digestion with pancreatic elastase. This view was supported by Labella (1957), who suggested that lipid was essential for maintaining the integrity of elastin and that elastolysis involved the release of lipid.

Other groups of workers believe that elastolysis is fundamentally a proteolytic process. Partridge & Davis (1955) pointed out that in elastic tissue the elastin fibres are intimately associated with collagen and mucopolysaccharide, but demonstrated that by a purification procedure based on repeated autoclaving with water at neutral pH, alternated with fine grinding, the basic fibres of elastic tissue could be separated in a form which maintained their original morphological structure and staining properties. Prepared in this way the elastin fibres contained only traces of carbohydrate and ester sulphate, and fractionation of the material after rendering it soluble by partial hydrolysis with dilute oxalic acid showed that it was homogeneous in amino acid composition. Lewis, Williams & Brink (1956) reported that crystalline pancreatic elastase hydrolysed other proteins in addition to elastin. The presence of the enzyme in the pancreatic juice of animals led to the suggestion that elastase should be regarded as a digestive proteinase. About the same time the significant observation was made that certain plant proteolytic enzymes will dissolve elastin. Thus Miyada & Tappel (1956) demonstrated the activity of ficin

* Part 4: Partridge & Davis (1958).

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and papain and Wang, Weir, Birkner & Ginger (1958) that of bromelin. Miyada & Tappel (1956) also reported the activity of a commercial proteolytic preparation of fungal origin, Rhozyme P-11. Lewis & Thiele (1957) showed by chromatography that their crystalline pancreatic elastase was heterogeneous and that, of the five components separated, only one component possessed elastase activity. Grant & Robbins (1957), using pancreatic elastase which had been partially purified by adsorption on to elastin, showed that it had strong proteolytic activity on proteins other than elastin. Naughton & Sanger (1958) extended the purification of pancreatic elastase by Lewis *et al.* (1956) to chromatographic fractionation and obtained on elution a well-defined peak which possessed all the elastase activity. When tested against the B chain of insulin it exhibited a wide range of peptide-bond specificity. The presence of two elastases in the pancreas has been reported by Dvonch & Alburn (1959), by the use of electrophoretic fractionation. Both these fractions had elastolytic and proteolytic activities.

It is clear that much of the confusion which exists in current views of the nature of elastin and the mechanism of elastolysis will be resolved only by a more detailed knowledge of the molecular architecture of the protein, and the final objective of the present work is to utilize elastolytic enzymes as tools for its stepwise degradation. However, to place such structural studies on an unequivocal basis it was considered necessary first to undertake a preliminary survey of the various enzyme preparations which have been reported as displaying elastolytic activity, with a preparation of elastin of established purity as substrate.

EXPERIMENTAL

Materials

Elastin. Extraction from bovine ligamentum nuchae was carried out as described by Partridge, Davis & Adair (1955). The carbohydrate content measured against a glucose standard was not more than 0·2% with the orcinol reagent (Sørensen & Haugaard, 1933) and 0·25% with anthrone (Yemm & Willis, 1954). The uronic acid content estimated by the carbazole method of Dische (1947) gave not more than 0·3% (as galacturonic acid). A colour corresponding to 0·14% of glucosamine was obtained by the Elson & Morgan (1933) reaction. The ash content was 0·14%.

Enzyme preparations. Pancreatin was obtained from Armour and Co. and Greeff and Co.; crude preparations of ficin were from Mann Research Laboratories, Merck and Co. and Nutritional Biochemical Corp.; papain was from British Drug Houses Ltd. and Harrington Bros. Ltd.; bromelin was from L. Light and Co. Ltd.; crystalline trypsin was from Armour and Co.; cereal α-amylases were from E. Gurr Ltd. and L. Light and Co. Ltd.; testicular hyaluronidase was from Benger Laboratories Ltd.; Rhozyme P-11 was from Rohm and Haas Co.; crystalline hog pancreatic and salivary α-amylases were generously given by Dr W. J. Whelan and bacterial chondroitinase was given by Dr A. G. Lloyd. Intracellular enzymes were extracted from kidney and spleen of a freshly killed ox by autolyses in acidic solution, followed by ammonium sulphate fractionation according to the instructions of De La Haba, Cammarata & Fruton (1955).

Carboxymethylcellulose. This was prepared as described by Petersen & Sober (1956).

Methods

Assay of elastase activity. The method was based on that of Naughton & Sanger (1958). Elastin (20 mg.) was weighed into a 10 ml. centrifuge tube and Congo red added (2 ml. of 0·02% solution in 0·1 M-sodium carbonate–HCl buffer, pH 8·8). No excess of dye remained in the supernatant after shaking at room temperature for 2–3 min. The enzyme solution was adjusted to the same buffer concentration and added to the dyed elastin suspension. More 0·1 M-sodium carbonate–HCl buffer (pH 8·8) was then added to give a final incubation volume of 5 ml. When cysteine was required 0·5 ml. of 0·25 M-cysteine solution (pH 8·8) was added, giving a final concentration of 25 mM. During incubation at 37·5° the elastin was kept in suspension by occasional shaking and the amount dissolved after 30 min. was measured by centrifuging and estimation of the colour in the supernatant with a Spekker colorimeter and filter no. 604.

In some experiments activity was also determined by the modification of Lewis *et al.* (1956) of the gravimetric method of Banga (1952).

A unit of elastase activity (e.u.) was defined as that amount of enzyme which dissolved 10 mg. of elastin in 0·1 M-sodium carbonate–HCl buffer, with or without cysteine (25 mM), when incubated at 37·5° for 30 min. The specific elastase activity (specific e.a.) was the number of units/mg. of enzyme.

Assay of proteolytic activity. This was based on the viscosity-decrease method of Whitaker (1957). A stock solution of gelatin was prepared by dissolving gelatin (2·5 g.) in water (50 ml.) with stirring and heating at 80°. After cooling, an equal volume of 0·2 M-phosphate buffer, pH 7·5, was added and mixed. To the enzyme solution (1 ml.) was added 0·1 M-phosphate buffer, pH 7·5 (1 ml.), and the mixture was brought to 37·5°. Gelatin solution (8 ml.) at 37·5° was added, the components were rapidly mixed and 5 ml. was transferred to an Ostwald viscometer (with a flow rate for water of 12·5 sec.) placed in a constant-temperature water bath at 37·5°. The flow rate was measured immediately and at regular intervals up to 10 min. When cysteine (25 mM) was required the enzyme solution was added to cysteine (0·25 M) in phosphate buffer, pH 7·5 (1 ml.). A blank was performed in the absence of enzyme solution.

A unit of proteolytic activity (p.u.) was defined as that amount of enzyme which decreased the specific viscosity of 100 mg. of gelatin to one-half when incubated in 0·1 M-phosphate buffer, pH 7·5, with or without cysteine (25 mM), after 10 min. at 37·5°.

Fractionation of ficin with ammonium sulphate. Ficin (20 g.) was stirred into 220 ml. of 0·1 M-acetate, pH 5·5, containing 20 mM-cysteine, for 30 min., the mixture was

centrifuged and the supernatant decanted. The residue was re-extracted with the acetate–cysteine mixture and centrifuged. The washings were combined with the supernatant and solid ammonium sulphate was added with stirring until the extract was brought to 30% saturation. The precipitate was removed by centrifuging and to the supernatant was added more ammonium sulphate until the saturation reached 40%. The precipitates obtained at 30, 40, 50 and 100% saturation were dissolved in salt solution and dialysed against frequent changes of distilled water containing 10 mM-cysteine. The euglobulin precipitates obtained during dialysis were collected by centrifuging, giving soluble and euglobulin fractions at each percentage of ammonium sulphate saturation.

Chromatographic fractionation of the 0–40% ammonium sulphate-soluble fraction from crude ficin. The column (23 cm. × 0·8 cm.) was packed with carboxymethylcellulose (Petersen & Sober, 1956) and equilibrated with 25 mM-sodium acetate buffer, pH 4·8, containing 5 mM-cysteine and 1 mM-ethylenediaminetetra-acetic acid. The same buffer was used to apply the ficin fraction (30 mg.) and to fill the mixing chamber (175 ml.) used for gradient elution. The column was developed by passing M-sodium acetate buffer, pH 4·8, into the mixing chamber at 2 ml./hr. and the effluent was collected in 4 ml. fractions. The protein content of each fraction was estimated by measuring E at 280 mμ.

Zone electrophoresis. The method used was essentially that of Svensson, Hagdahl & Lerner (1957), in which a vertical column is stabilized by a sucrose-density gradient. The column was 42 cm. high × 3·2 cm. diameter; buffer, 30 mM-sodium barbitone, pH 8·67; the sucrose concentration varied from 8% (w/v) at the point of injection of the sample to 30% (w/v) at the outlet. The solution of ficin (52 mg. in 10 ml. of sucrose–buffer of the appropriate density) was injected into the column and migration allowed to proceed in the downward direction overnight at room temperature (6·7 v/cm.; 20 mA). Fractions (10 ml.) were then collected by discharge from the outlet tube at the bottom of the column and were estimated for protein by measuring the extinction with the Folin–Ciocalteu reagent (Lowry, Rosebrough, Farr & Randall, 1951).

RESULTS

The measurement of elastase activity is a matter of some difficulty owing to the complexity of the kinetics of the reaction. Banga (1952) defined a unit of elastase activity as the amount of enzyme which dissolved 5% of a specified quantity of elastin in 30 min. under defined conditions, and difficulties due to the lag phase of the reaction were avoided by adjusting the dilution of the enzyme until the assay tube contained a quantity close to one unit. In our hands this scheme appeared to give reproducible results when assaying a single sample of enzyme, but when enzymes from different sources are to be assayed there is an obvious danger of error if the preparation of elastin should contain a small amount of foreign protein as impurity. Accordingly for the purposes of the present work the elastase unit was redefined as the amount of enzyme which dissolved 50% of a 20 mg. portion of elastin in 30 min. under the conditions specified.

Assay of proteolytic enzymes. The elastolytic and proteolytic activities of crude commercial preparations of various enzymes which are known to hydrolyse proteins are summarized in Table 1. The report by Miyada & Tappel (1956) that Rhozyme P-11 hydrolysed elastin was not confirmed, despite the proteolytic activity exhibited. However, in agreement with these workers and Wang *et al.* (1958), it was shown that ficin, papain and bromelin possess strong elastolytic and proteolytic activities. These plant enzymes require activation by a reducing agent, e.g. cysteine, and in its absence there was a marked decrease in activity towards elastin and gelatin. The two commercial pancreatin preparations tested were very much less active than the plant enzyme both in elastolytic and proteolytic effect. It is to be noticed that the ratio of elastase to proteolytic activity in all the preparations used did not vary over a wide range. In contrast with these results, the preparations of crystalline trypsin, chymotrypsin and pepsin were either completely inactive or showed a slight dissolution of elastin after several days.

Assay of mucolytic enzymes. Pepler & Brandt (1954) claimed that a sample of chondrosul-

Table 1. *Elastolytic and proteolytic activities of proteolytic enzymes*

The definition and method of estimation of specific e.a. and specific proteolytic activity (p.a.) are given under Methods. Cysteine (0·025 M) was present in incubation mixtures with ficin, papain and bromelin.

Enzyme	Commercial source	Specific e.a.	Specific p.a.	Specific e.a./ specific p.a.
Ficin	Merck and Co.	0·186	87·0	0·00214
	Mann Research Laboratories	0·218	87·6	0·00249
	Nutritional Biochemicals Corp.	0·143	83·3	0·00172
Papain	British Drug Houses Ltd.	0·50	77·0	0·00649
	Harrington Bros. Ltd.	0·50	84·0	0·00596
Bromelin	L. Light and Co. Ltd.	0·21	44·0	0·00477
Pancreatin	Armour and Co.,	0·007	1·25	0·00560
	Greeff and Co.	0·004	0·85	0·00471
Rhozyme P-11	Rohm and Haas Co.	0	12·0	—

phatase from *Proteus vulgaris* dissolved elastic fibres. In the present assays a preparation of dried cells from *P. vulgaris* (National Collection of Type Cultures, no. 4636), which contained both chondrosulphatase and chondroitinase, was tested against purified elastin but was found to be inactive. A sample of pure chondroitinase from *P. vulgaris* (Dodgson & Lloyd, 1957) showed no proteolytic activity, and also failed to digest elastin. In view of the report by Yokota (1957) that a commercial preparation of α-amylase possessed elastolytic activity, an assay was made of a number of samples of α-amylase extracted from different sources. Two commercial cereal preparations and crystalline salivary α-amylases showed no activity towards elastin or gelatin. However, a crystalline preparation of hog pancreatic α-amylase showed weak elastolytic activity but this disappeared when the enzyme was recrystallized. A purified sample of testicular hyaluronidase (Benger Laboratories Ltd.) had no elastolytic or proteolytic activity.

Kathepsins. The presence in mammalian tissues of intracellular proteolytic enzymes known collectively as kathepsins has been known for many years. By the method of De La Haba *et al.* (1955) for extraction of these enzymes from fresh kidney and spleen, fractions were obtained which possessed proteolytic activity when assayed in the presence of reducing agents but did not digest elastin either in the presence or the absence of cysteine.

Fractionation and purification of ficin. The commercial preparations are described as the products of drying fig latex, and the question arises whether the elastolytic activity observed is a property of the proteolytic enzyme which forms a large part of the product or whether it is due to the presence of a separate specific elastase. Accordingly, one of the preparations (Merck and Co.) was extensively fractionated first by ammonium sulphate precipitation and then by zone electrophoresis. A highly purified product was then isolated by chromatographic fractionation with a column of carboxymethylcellulose. Table 2 shows the results of fractionation with ammonium sulphate, and it will be observed that the distribution of elastase activity follows closely that of proteolytic activity as measured by following the decrease of viscosity of gelatin. Most of the active enzyme is precipitated at 50% saturation with ammonium sulphate or below, and activity is associated with the pseudoglobulin fraction which is soluble in water after dialysis.

From the data in Table 2 the elastolytic and proteolytic activity appeared to be concentrated in the 30 and 40% ammonium sulphate-soluble

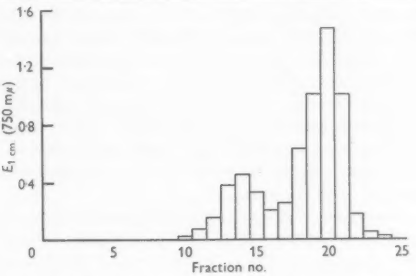

Fig. 1. Zone-electrophoresis pattern given by 52 mg. of ficin (water-soluble fraction precipitated at 40% ammonium sulphate saturation) with a sucrose-density-gradient column. Protein concentrations of fractions were estimated from E at 750 mμ after addition of Folin–Ciocalteu reagent.

Table 2. *Fractionation of ficin with ammonium sulphate*

All estimations were carried out in the presence of cysteine (25 mM). The definitions of p.u. (proteolytic unit), specific p.a. (specific proteolytic activity), e.u. (elastase unit) and specific e.a. are given under Methods.

Fraction	Dry wt. (g.)	Total p.u. ($\times 10^{-3}$)	Specific p.a.	Total e.u.	Specific e.a.	Specific e.a./ specific p.a.
Commercial ficin	20·000	1820	91	3720	0·186	0·00205
Insoluble in acetate	0·245	0	0	0	0	0
30% satn. $(NH_4)_2SO_4$ ppt.						
Soluble in water	1·500	667	445	1480	0·99	0·0022
Euglobin	0·694	136	196	417	0·61	0·0031
40% satn. $(NH_4)_2SO_4$ ppt.						
Soluble in water	0·316	240	760	277	0·88	0·0012
Euglobin	0·087	13	154	35	0·41	0·0027
50% satn. $(NH_4)_2SO_4$ ppt.						
Soluble in water	2·120	1074	506	930	0·44	0·0087
Euglobin	0·980	85	87	202	0·21	0·0024
100% satn. $(NH_4)_2SO_4$ ppt.						
Soluble in water	1·570	194	123	175	0·11	0·0089
Euglobin	0·144	11	74	35	0·24	0·0033

fractions and these were combined for further fractionation by zone electrophoresis with a sucrose-density-gradient column. The resultant curve, showing two protein peaks, is given in Fig. 1. Fractions 14 and 20 were tested for elastolytic and proteolytic activities. All the activity was associated with the slower-moving fraction 20, the ratio of elastolytic to proteolytic activity being 0·00163 compared with 0·00256 for the starting material. The peak represented by fraction 14 possessed no elastolytic or proteolytic activity.

The same ammonium sulphate fraction was further fractionated by gradient elution with a carboxymethylcellulose column. The buffer system contained cysteine and ethylenediaminetetraacetic acid and the elution was conducted by increasing the concentration of the sodium acetate buffer, the pH being maintained at 4·8 throughout. The resulting curve of protein concentration is shown in Fig. 2. Fractions 3, 14, 18, 20, 28, 38 and 48 were dialysed against distilled water containing 5 mM-cysteine and 1 mM-ethylenediaminetetraacetic acid and then tested for elastolytic and proteolytic activities. Fractions 3 and 48 were devoid of both activities, 28 and 38 showed slight activity but fractions 14 and 20 were both highly active. In both fractions the ratio of elastolytic to proteolytic activity was the same.

Pancreatic elastase. Although pancreatic elastase is not regarded as an SH-dependent enzyme (Naughton & Sanger, 1958) it was observed that the addition of cysteine (25 mM) to the incubation mixture increased the activity of crude preparations of the enzyme two- or three-fold. The possibility that pancreatin contains a second, SH-active elastase was tested by fractionating a preparation of crude pancreatin and comparing the properties of the fractions. An extract of commercial pancreatin in sodium acetate buffer was fractionated with ammonium sulphate according to the directions of Lewis *et al.* (1956). The fractionation results are shown in Table 3. The most active material was the water-insoluble (euglobulin) fraction obtained after dialysis of the fraction

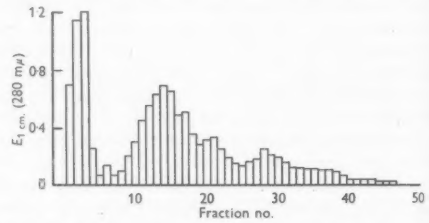

Fig. 2. Ultraviolet-absorption diagram from 30 mg. of ficin (water-soluble fraction precipitated at 40% ammonium sulphate saturation) obtained after elution with a concentration gradient of acetate buffer, pH 4·8, with a carboxymethylcellulose column.

Table 3. *Fractionation of pancreatin with ammonium sulphate*

For explanation of abbreviations see Table 2.

Fraction	Dry wt. (g.)	Total p.u. ($\times 10^{-3}$)	Specific p.a.	Total e.u.	Specific e.a.	Specific e.a./ specific p.a.
Starting material	100	120·0	1·20	690	0·007	0·0059
Insoluble in acetate	4·08	15·2	3·72	123	0·030	0·0081
45% satn. $(NH_4)_2SO_4$ ppt.						
Soluble in water	1·04	25·6	24·60	213	0·204	0·0083
Euglobin	0·10	3·2	32·40	200	2·000	0·0618
100% satn. $(NH_4)_2SO_4$ ppt.	0·43	4·2	9·80	5	0·120	0·0122

Table 4. *Effect of activators and inhibitors on purified pancreatic elastase*

For explanation of abbreviations see Table 2.

Molarity of carbonate buffer (pH 8·8)	Addition	Specific e.a.	Effect
0·1	None	2	—
0·1	Cysteine (25 mM)	5	Activates × 2·5
0·1	Di*iso*propyl phosphorofluoridate (0·1 mM)	0	Complete inhibition
0·1	H_2O_2 (30 mM)	4	Activates × 2·0
0·1	Iodoacetic acid (20 mM)	2	No effect
0·05	None	7	Activates × 3·5
0·05	Cysteine (25 mM)	7	Activates × 3·5; cysteine has no effect
0·1	NaCl (0·1 M)	0·25	Inhibition
0·1	NaCl (0·1 M) + cysteine (25 mM)	0·60	Cysteine reduces inhibition

precipitated at 45% ammonium sulphate concentration. The specific activity of this fraction was 286 times that of the starting material. The activity of this highly enriched fraction was increased by the presence of cysteine in the same ratio (1:2·5) as was observed with crude pancreatin and the other less active fractions. It was therefore concluded that cysteine activation is a property of pancreatic elastase itself and is not due to the presence of a second elastolytic enzyme. As would be expected from the known presence of several proteolytic enzymes in crude pancreatin, the ratio of elastolytic to proteolytic activity of the fractions varied over a wide range.

The effect of activators and inhibitors on the euglobulin fraction is reported in Table 4. Iodoacetic acid (20 mM), an inhibitor of SH-dependent enzymes, had no effect and hydrogen peroxide behaved like cysteine in producing marked activation. The complete inhibition by di*iso*propyl phosphorofluoridate at 0·1 mM concentration reported by Naughton & Sanger (1958) was confirmed. This inhibitor has no effect on SH-dependent enzymes and the complete loss of activity in its presence tells strongly against the possibility of the existence of a separate SH-elastase in pancreatin. All the elastase assays described above were performed in the presence of 0·1 M-carbonate buffer, but it was found that if the buffer concentration was reduced to 50 mM there was a 3·5-fold increase in activity. At this lower ionic strength cysteine was found to have no effect on activity, thus confirming the report of Lewis *et al.* (1956), who used a low buffer concentration in their assays and observed no increase in activity with added cysteine. The addition of sodium chloride (0·1 N) to an incubation mixture containing 0·1 M-carbonate caused marked inhibition of elastase activity. The inhibition, however, was decreased in the presence of cysteine.

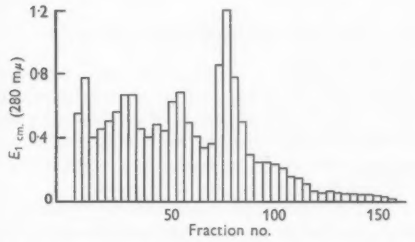

Fig. 3. Ultraviolet-absorption diagram from 25 mg. of pancreatin (euglobulin fraction precipitated at 45% ammonium sulphate saturation) obtained after elution with a concentration gradient of acetate buffer, pH 4·8, with a carboxymethylcellulose column.

Further purification of the euglobulin fraction of pancreatic elastase was obtained by fractionation on a carboxymethylcellulose column (Naughton & Sanger, 1958), gradient elution with acetate buffer, pH 4·8, being used. Fig. 3 shows the presence of five protein peaks, nearly all of the activity being associated with the slowest moving peak (fraction 78). The specific elastase activity of the material from this peak was 5·0, showing a purification factor of 714, based on the activity of crude pancreatin. The specific proteolytic activity of the preparation was 34·4.

DISCUSSION

In carrying out this survey of elastolytic enzymes it was considered of importance that the substrate used should be one of established purity. By this it is meant that the preparation of elastin should represent the major structural element of elastic tissue, and that it should be substantially free from all contaminating material, arising from other components of connective tissue, that can be removed by physical means. We consider that the preparation of elastin described by Partridge, Davis & Adair (1955) satisfied these requirements in that: (i) the method of isolation avoids the use of hydrolytic reagents such as alkalis and acids; (ii) the fibrous preparation has the morphological structure and staining reactions of the parent tissue; (iii) it is substantially free from collagen and carbohydrate; (iv) on fractionation after partial hydrolysis the fractions have substantially the same amino acid analysis. Elastin prepared in this way may be regarded as a fibrous protein in a relatively high state of purity, but, as pointed out by Partridge & Davis (1955), only 95% of the protein dry weight could be accounted for by amino acid analysis and it is possible that nitrogenous or non-nitrogenous residues other than amino acids may be involved in its structure.

On digestion with pancreatic elastase Banga (1951), using the formol-titration and van Slyke methods of estimation, failed to detect release of α-amino groups. However, Partridge & Davis (1955), using the more sensitive fluorodinitrobenzene technique of Sanger (1945), observed release of amino groups during elastolysis and concluded that the mechanism was essentially a proteolytic one. Purified pancreatic-elastase preparations have a wide range of proteolytic activity and hydrolyse heat-treated collagen (Banga, 1953), haemoglobin (Patridge & Davis, 1955), fibrin, albumin, casein and soya-bean protein (Lewis *et al.* 1956). Esterase activity has also been demonstrated towards acetyl-L-tyrosine ethyl ester (Grant & Robbins, 1957) and rennet activity (Lewis *et al.* 1956).

The present work confirms the finding of Miyada & Tappel (1956) and Wang *et al.* (1958) that certain proteolytic enzymes of vegetable origin have a high elastolytic activity, but no elastolytic or proteolytic activity was detected in any of the mucolytic enzymes tested. A sample of α-amylase prepared from pancreas extract did indeed dissolve elastin, but this activity disappeared when the enzyme was recrystallized and it was concluded that the activity was the result of contamination with pancreatic elastase. In this connexion it should be mentioned that the presence of an unidentified proteolytic enzyme in crystalline preparations of pancreatic α-amylase has already been reported by Bernfeld (1951).

In all enzyme preparations tested elastolytic activity was always associated with proteolytic activity. The ratio of these activities was about the same in crude preparations of bromelin, ficin and papain. With ficin the crude commercial product was fractionated and purified, and a fraction was obtained by chromatography on a carboxymethylcellulose column in which the specific activity was sevenfold that of the starting material. All the major active fractions and the finally purified product had about the same ratio of elastolytic to proteolytic activity and it is therefore concluded that the elastase of fig latex is identical with the enzyme responsible for the larger part of the proteolytic activity.

The purification of pancreatic elastase was based on the procedure of Naughton & Sanger (1958). During the course of the assays the observation was made that under some conditions of pH and ionic strength the enzyme was markedly activated by the presence of reducing agents such as cysteine, suggesting the presence of an SH-enzyme. However SH-inhibitors such as iodoacetic acid had no effect, and hydrogen peroxide, instead of decreasing the activity of the enzyme, appeared to enhance it somewhat. In agreement with Naughton & Sanger it was found that di*iso*propyl phosphorofluoridate inhibited the elastase activity and thus there appears little doubt that the active centre of pancreatic elastase is similar to that of trypsin and chymotrypsin (cf. Dixon, Neurath & Pechere, 1958). The enhanced activity due to cysteine and hydrogen peroxide may then be due to the rupture of an –SS– bond elsewhere in the molecule. It is considered possible that the active –SS– bond may constitute an intermolecular link, and in this case the presence of cysteine or hydrogen peroxide would reduce the number of dimers present. A decrease in salt concentration from 0·1 M to 50 mM results in activation of the same order and this may also be due to a similar disturbance of an association equilibrium. This indication of association phenomena could account in part for the complex kinetics of elastase action observed by many workers, but in considering this question the possibility of the presence of an inactive zymogen (Grant & Robbins, 1955) should not be forgotten.

The results of the survey as a whole show that whereas all non-proteolytic enzymes so far tested are inactive towards elastin several well-characterized proteolytic enzymes have a high activity. The supposition that there is a specific non-proteolytic 'elastase' which dissolves the fibrous protein and thus renders it susceptible to proteolytic hydrolysis by a second enzyme is scarcely tenable in view of the failure of the fractionation experiments with ficin and pancreatin to disclose such a system. Since the specificity of proteolytic enzymes as a whole seems to be associated with the rupture of specific peptide links, it may well be asked why some proteolytic enzymes are active as elastases and others only slightly active or not at all. It has already been pointed out (Newman, 1949; Partridge & Davis, 1955) that elastin has an unusual amino acid composition, being very high in residues with hydrocarbon side chains, particularly valine, and very low in acidic and basic amino acids. There is also evidence that the fibrous protein has a cross-linked structure (Partridge, Davis & Adair, 1955). It is possible that in order to split a sufficient number of peptide links to disrupt the net-like structure of the protein an enzyme displaying a wide peptide-bond specificity is essential. Enzyme preparations such as crystalline trypsin may fail because they attack a too small proportion of the links. It is also probable that in some cases a proteolytic enzyme may show little activity because of steric factors concerning its size, shape and position of active centre; thus both the activity of pancreatic elastase, and its adsorption by elastin, is greatly enhanced if the elastin is pretreated with boiling dilute sodium hydroxide (Grant & Robbins, 1957). This treatment would be expected to open up the structure of the network by the rupture of a proportion of the links.

SUMMARY

1. A number of proteolytic and mucolytic enzymes have been assayed for elastolytic activity with a preparation of elastin of established purity as substrate. This was prepared from bovine ligamentum nuchae; it contained less than 0·3% of carbohydrate and was free from collagen.

2. In addition to pancreatic elastase, three proteolytic enzymes, papain, ficin and bromelin, were found to have a high activity towards elastin. Crystalline trypsin, chymotrypsin, pepsin, Rhozyme and preparations of kathepsin from kidney and spleen were found to be either totally inactive or very slow.

3. Preparations of bacterial chondrosulphatase, purified chondroitinase and testicular hyaluronidase showed no activity.

4. Preparations of α-amylase from saliva and cereal seeds were inactive but a crystalline sample of pancreatic α-amylase showed activity. This disappeared on further recrystallization, and it is concluded that the elastolytic activity of α-amylase reported in the literature is due to contamination with pancreatic elastase.

5. Various commercial preparations of ficin all showed the same ratio of elastolytic to proteolytic activity as measured by viscosity reduction of gelatin. This same ratio was maintained throughout fractionation and purification of commercial dried fig latex. It was concluded that 'fig-latex elastase' is identical with the major proteolytic enzyme in preparations of 'ficin'.

6. The results of the survey confirm the view that elastolysis is the result of proteolytic activity. The failure of certain well-characterized proteolytic enzymes such as trypsin and chymotrypsin to dissolve elastin is attributed either to their failure to penetrate the cross-linked structure of elastin or to the very restricted peptide-bond specificity exhibited by these enzymes.

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The Formation of Phenolic Substances in *Eucalyptus gigantea* and *Eucalyptus sieberiana*

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It has been pointed out (Hillis, 1956; Hathway, 1958) that resins and kinos produced in the cambial region contain components which are absent from heartwood of the species which produced them. For these and other reasons, it was suggested that the phenolic substances in bark and wood were produced elsewhere. It was further suggested that, in some instances, the leucoanthocyanins were the precursors of heartwood extractives (see also Bate-Smith & Swain, 1953; King & White, 1957; Roux, 1958; Roux & Evelyn, 1958). Recent information obtained with the aid of a staining technique (Hillis, 1958) indicated that the leucoanthocyanins in some eucalypts were synthesized *in situ*. A

preliminary study of the phenolic substances from the cambial and woody tissues of other eucalypts supported this view of formation *in situ* (Hillis & Carle, 1958). The present work was undertaken to gain more information about the precursors of the phenolic substances in the heartwood of *Eucalyptus* spp. and their site of biosynthesis.

Eucalyptus gigantea and *Eucalyptus sieberiana* were investigated as their pale-coloured heartwoods contain small amounts of leucoanthocyanins but larger amounts of other phenolic substances. An examination of the latter would show whether they interfere with the staining technique (Hillis, 1958) for leucoanthocyanins, as reports (Jurd, 1957; Grassman, 1958) indicate that other substances could do so. As eucalypt kinos contain large amounts of leucoanthocyanins, the contrast in their chemical composition with that of heartwood extractives may indicate the role of leucoanthocyanins. Kino is usually formed when the cambium of *Eucalyptus* trees (and of members of some other genera) is damaged. Kino is found in veins or pockets according to the severity of the injury and the species. In veins, the accretions of kino are separated by radial bridges of woody tissue, and rarely interfere with the conversion of sapwood into heartwood.

EXPERIMENTAL

Materials

Wood and phloem samples. The samples of *E. gigantea* were taken from freshly felled logs (Table 1), wrapped in polythene bags and examined within 3 weeks of collection. Log 4 was collected from a 100-year-old *E. sieberiana* (3 ft. diam.) tree grown in north-eastern Victoria, and log 5 from a 5-year-old *E. sieberiana* (2 in. diam.) grown in Melbourne.

Kino. Viscous *E. gigantea* kino was taken from the heartwood in log 3. The *E. sieberiana* kino was collected from experimental trees (see below).

Study of kino formation

The two experimental *E. sieberiana* were 5 years old and in a vigorous state of growth. The experiments were carried out from mid-summer to early autumn, when the cambium was still active. One tree was polled at 4 ft. above ground level, the saw cut being 3 in. above the two remaining branches. Incisions of different types and at different heights above ground level were made on the other tree. In all cases the cambium was exposed or damaged.

Epicormic shoots began to form below the highest damaged areas. The polled tree formed twenty-two vigorous shoots and the other tree formed thirteen shoots. One month after injury the epicormic shoots on the polled tree were about 5 in. long and bore three or four leaves; traces of kino were then evident in some of the cuts and the daily production increased as the shoots lengthened (up to 3·25 ft. in 9 weeks). This sequence of events had been observed in a 4-year-old tree which had been polled in the previous season.

The amount of kino produced by the different types of injuries varied; 1·2 g. (dry wt.) of kino was collected from the top of the polled tree and 2·9 g. from the other injuries. Some of the knife cuts healed over and did not exude kino, and only traces of kino were obtained from a slash wound, the periphery of which was covered by callus tissue. The kino exuded from the horizontal uppermost cut of the triangular-shaped incisions.

When freshly exuded, the kino of *E. sieberiana* was a pale tan honey-like material which dried to a glassy solid. After standing for 3–4 weeks the colour became increasingly red.

Chromatographic methods

The samples (or methanol extracts of fresh tissues) were dissolved in methanol, methanol–water (1:1, v/v) or dimethylformamide. Solutions of the samples were applied to Whatman filter papers nos. 1 or 3, by repeated applications in a warm air stream. A reference compound (phloroglucinol, gallic acid or cyanidin) was used on each one-way chromatogram and phloroglucinol added to every alternate two-way chromatogram. The chromatograms were developed at 20°.

Developing solvents. Two-way chromatograms were developed by an ascending method employing (1) 6% acetic acid followed by (2) butan-1-ol–acetic acid–water (6:1:2, by vol.) after drying in air for 4 hr. Both solvents travelled about 11·5 in. One-way chromatograms were developed with the following solvents: (3) HCl–acetic acid–water (3:30:10, by vol.) (Bate-Smith, 1954); (4) butanol–acetic acid–water (10:4:2, by vol.) (Schmidt, Hensler & Stephan, 1957); (5) butanol–acetic acid–water–glycol (4:1:5:1, by vol.) (Schmidt & Lademann, 1951*a*); (6) ethyl acetate–acetic acid–water–ethanol (3:1:3:0·5, by vol.) (Schmidt & Schmidt, 1952); (7) formic acid–HCl–water (5:2:3, by vol.) (Harborne, 1958*a*); (8) butanol–HCl–water (7:2:5, by vol.) (Abe & Hayashi, 1956).

Table 1. *Description of samples of* Eucalyptus gigantea

	Log 1	Log 2	Log 3
Age of tree (years)	300	300	100
Collected in	Northern Tasmania	Northern Tasmania	North-eastern Victoria
Diam. of log (ft.)	3·5	4·5	3
Location of heartwood samples:			
(*a*) Outer	Outer 5 in.	Outer 4 in	Outer 2 in.
(*b*) Inner	Adjacent to pith	Adjacent to pith	—

Colour reactions. After the chromatograms had been air-dried for 2–5 hr. the resolved constituents were detected by the colour given under the following conditions: (*a*) in daylight; (*b*) under ultraviolet light; (*c*) under ultraviolet light whilst fuming with ammonia (sp.gr. 0·88). The chromatograms were then sprayed with the following chromogenic reagents: (*d*) ferric chloride–potassium ferricyanide (White, Kirby & Knowles, 1952), for phenols; (*e*) diazotized *p*-nitroaniline–sodium acetate (Swain, 1952), for phenols; (*f*) vanillin–HCl (Bate-Smith & Swain, 1953), for flavans with phloroglucinol nucleus; (*g*) 5% potassium cyanide solution (Young, 1883), for gallic acid; (*h*) sodium periodate and aniline (Yoshida & Hasegawa, 1957), for shikimic acid; (*i*) bromocresol green (Lugg & Overell, 1947), for acidic compounds; (*j*) aniline–phthalic acid (Partridge, 1949), for aldose sugars; (*k*) phloroglucinol–HCl (Wiesner, 1878), for lignin.

Estimation of ellagic acid

Portions (20 g.) of the fresh samples were split and soaked in methanol for 48 hr. with two changes of solvent. The samples were then air-dried, ground in a small Wiley mill to 60-mesh and extracted in a Soxhlet apparatus for 20 hr. The two extracts were combined. Measured portions were hydrolysed by heating with 2N-HCl under reflux on a boiling-water bath for 6 hr. Known fractions of the unhydrolysed and hydrolysed extracts were suitably diluted with dimethylformamide. The ellagic acid in these samples was estimated by comparing the area and the intensity of fluorescence of the spot with those of standards of pure ellagic acid after chromatography in solvent 3 (Hillis & Carle, 1959).

Examination of leucoanthocyanins

The anthocyanidin produced by heating a solution of the kinos in methanol–water (1:1, v/v) with butanol–HCl (100:5, v/v) (Swain & Hillis, 1959) was estimated as delphinidin from the absorption at the maximum wavelength with the constants of Schou (1927). The R_F values of the anthocyanidins in solvents 3, 7 and 8 were obtained on examination of the product given by heating kino (0·7 g.) with butanol–HCl (30 ml.) on a boiling-water bath for 30 min. The R_F values of the major anthocyanidin were identical with those of delphinidin, and traces of cyanidin (less than one-tenth of delphinidin) were present. Purified delphinidin was obtained for spectral examination by streaking this solution on no. 3 paper, developing in solvent 3, and extracting the band at R_F 0·3 at room temperature in darkness with methanol containing 0·01% of conc. HCl. An absorption maximum of 561 mμ was obtained (see Harborne, 1958*b*). The colour reactions were observed on material obtained by displacing the above reaction products in butanol (5 ml.) into 1% (v/v) HCl (2 ml.) by means of benzene (20 ml.) and extracting impurities in the aqueous layer with ethyl acetate (3 × 5 ml.). After extraction of the remaining aqueous layer repeatedly with benzene, the colour reactions with pentanol–sodium acetate and the cyanidin reagent (Robinson & Robinson, 1931) were observed and found to be those of delphinidin.

The products obtained by heating woody tissues or their extracts with butanol–HCl were purified as above and the red was extracted with pentanol. Portions of the fractions from the methanol extract of heartwood were heated with butanol–HCl as above and the colours observed.

Degradation of kino

Samples of kino (1 g.) were heated with 3N-HCl (20 ml.) on a boiling-water bath for 40 min. and the aqueous layer was extracted with ether in a liquid–liquid extractor for 16 hr. The extract was examined by means of two-way cochromatography in solvents 1 and 2, and colour reactions (*b*)–(*g*) and (*j*), and ellagic acid, gallic acid and phloroglucinol were shown to be present.

Extraction of constituents from wood

Fresh samples of outer (1·5 kg. dry wt.) and inner (2·0 kg. dry wt.) heartwood of *E. gigantea* were taken in the form of 1 in. cubes from log 1. They were processed separately in the same manner as that used in the estimation of ellagic acid (q.v.) except that the extraction periods were longer.

Fractionation of extracts

The combined methanol extracts from each sample were vacuum-concentrated (to approx. 500 ml.), water (500 ml.) was added and they were centrifuged to remove the insoluble materials. The liquor was extracted with light petroleum (b.p. 40–60°) for 6 hr. in a liquid–liquid extractor, then with ethyl ether for 4 × 8 hr. and finally with ethyl acetate for 6 × 8 hr. with regular changes of solvent. A further amount of buff-coloured insoluble material was centrifuged from the remaining black liquor.

A portion of the methanol-extracted outer heartwood was extracted ten times with 2N-NaOH at 70° and the filtrate was immediately neutralized.

Examination of fractions

Ethyl ether-soluble fraction. Two-way chromatograms were run (solvents 1 and 2, colour reactions *a*–*e*). Only about one-third of this fraction had R_F values > 0·5 in solvent 2. It was further fractionated by passing the material which was soluble in ether saturated with water through a column (prepared by the method of Bradfield & Penney, 1947) of silica gel (L. Light and Co. Ltd.; 'chromatographic grade'; purified by the method of Hathway, 1958). The column was developed with ether saturated with water, and the eluent after drying was washed five times with small amounts of ether; the insoluble material recrystallized three times from water to yield pale-brown needles. This component (no. 10, Fig. 1) was identified as gallic acid by its mixed m.p. (215°) with authentic material, its R_F values in solvents (1)–(4) and colour reactions (*d*), (*e*) and (*g*), when compared with authentic material, and its ultraviolet-absorption spectra in ethanol and ethanol–sodium acetate (Jurd, 1956).

The mother liquor contained several components (2–7) with similar R_F values (Fig. 1); these reacted weakly with colour reagent (*d*), their R_F in butanol–water was greatly reduced when borate paper was used, and components 2–7 and 13 were unchanged after boiling with 2N-H_2SO_4 for 1·5 hr. Attempts to separate the components on cellulose columns with different solvents failed to purify them to permit identification. Chromatographic examination showed that the more common polyphenols and aromatic acids were absent.

Ethyl acetate-soluble fraction. The components present had R_F values less than 0·5 in solvent 2. A portion of the vacuum-evaporated mixed extract (5·0 g.) was mixed with

dry cellulose powder (0·5 g.), then water (5 ml.) and a few drops of ethanol were added and the mass was stirred until a slurry was obtained. The slurry was carefully added to the top of a 1·25 in. diam. × 10 in. cellulose column. The column had previously been prepared by pouring a thick slurry of cellulose (Solka Floc, Brown Co., Mass., U.S.A.; purified by the method of Campbell, Work & Mellanby, 1951) and dry acetone into the chromatography tube with its tap open. When the cellulose had settled to a height of 10 in., the supernatant acetone and suspended cellulose were removed from the top and water (about 2 l.) was allowed to percolate through the column. After the addition of the sample to the column, water was added to maintain a head of 3 ft., 120 × 10 ml. fractions were collected over a period of 7 hr. and each fraction was chromatographed in 6% acetic acid. The contents of the tubes were mixed as follows: group A, tubes 1 and 2; B, tubes 3–7; C, tubes 9–13; D, tubes 17–30; E, tubes 33–53; F, tubes 57–120; NaCl was added to saturation. Then they were completely extracted (for periods varying from 2 days for group F to 2 weeks for group A) with ethyl acetate in a liquid–liquid extractor. After drying (Na_2SO_4) and concentration, the extract was added to five times its volume of warm hexane, and the precipitate washed with hexane and dried at 100°. Groups A and F yielded almost pure components 28 and 18 (Fig. 1) respectively whereas groups B–E contained components 20–26 in different stages of purity.

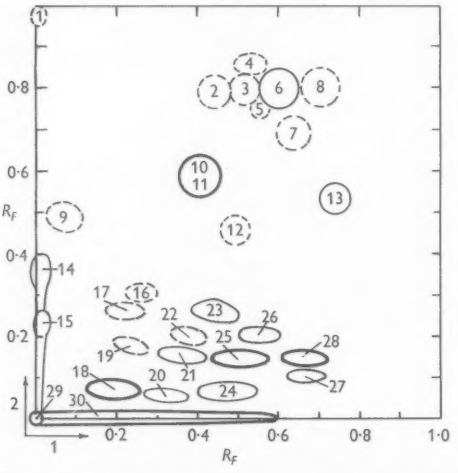

Fig. 1. Chromatogram showing major phenolic substances in the woody tissues of *E. gigantea*. The chromatogram was run first in 6% acetic acid followed by butanol–acetic acid–water (6:1:2, by vol.). On the diagram the relative intensity is shown by: - - - -, weak; ——, medium; ——, strong. Substances identified are: spot 10, gallic acid; 11, (+)-catechin (sapwood only); 14, ellagic acid; 22, corilagin (traces); 25, juglanin. Spots 10, 12 and 14–30 gave a blue with $FeCl_3$–$K_3Fe(CN)_6$; spots 6 and 13 gave a straw-yellow, spots 4, 5 and 7 an orange, spots 3 and 10 a yellow changing to black and spot 1 a purple colour with diazotized *p*-nitroaniline; spot 2 had a water-green, spot 9 a yellow u.v. fluorescence, and spots 4, 5 and 8 a light-blue u.v. fluorescence after fuming with ammonia.

These components gave a strong blue with iron salts and a pink to brown with 2N-NaOH, indicating the presence of vicinal trihydroxyl groups. As no colour change took place when the two-way chromatogram was sprayed with bromocresol green it is evident that they were not acidic. Samples of groups B–F were separately hydrolysed with 2N-H_2SO_4 for 2 hr. and the samples taken at intervals of 20 min. were chromatographed in solvents 1 and 2. Ellagic acid and gallic acid and glucose, apart from traces of a phenolic substance R_F 0·85/0·35 (solvents 1 and 2), were the only substances shown to be present by means of the colour tests (*a*)–(*e*), (*g*) and (*j*). They were identified by their R_F values when compared with authentic materials. When the ethyl acetate-soluble fraction (components 18–28) was hydrolysed with aq. 5% H_2SO_4, ellagic acid (34% yield) and glucose were separated by the method of Jurd (1958). The identity of the glucose was confirmed by the separate and mixed m.p. (205°) of the glucosazone.

Components 22 and 25 were identified as corilagin (Schmidt & Lademann, 1951*b*; Schmidt, Schmidt & Herok, 1954) and juglanin (Jurd, 1958) respectively, by cochromatography on two-way chromatograms with solvents 1 and 2, comparison with authentic samples in solvents 4, 5 and 6, together with colour reactions (*b*)–(*e*). Traces only of corilagin were present. The R_F values of corilagin and juglanin reported by Jurd (1958) are higher than those in Fig. 1. However, we have found that under our conditions the R_F values in solvents 1 and 2 of these phenols from the walnut were very similar to those of components 18–28.

Insoluble material. Chromatographic examination showed that the insoluble fraction consisted almost entirely of ellagic acid (see also Hillis, 1953). It was identified as described above by its R_F values and colour reactions when compared with authentic material, and also by the separate and mixed m.p. (245–246°) of its tetracarbethoxy derivative (Feist & Bestehorn, 1925; Reichel & Schwab, 1942). Also present were traces of a phenolic material immobile in all the solvents tried and with a bright emerald-green ultraviolet fluorescence.

RESULTS

Phenolic substances in the heartwood of Eucalyptus gigantea

The percentage (on a dry-weight basis) of the fractions of the methanol extractives from the outer and inner heartwoods respectively of log 1 were as follows: petroleum-soluble, —, 0·06; ethyl ether-soluble, 0·61, 0·52; ethyl acetate-soluble, 3·15, 1·78; insoluble, 1·00, 0·66. A large portion of the methanol extractives (see Table 2) was a dark-brown material (30) which could not be resolved chromatographically.

Petroleum-soluble fraction. When chromatographed this fraction occupied the position of component 1 (Fig. 1, Table 3). In earlier work on *E. sieberiana* (Hillis & Carle, 1958) the purple reaction (with spray *e*) was given only by the extracts of cambium, particularly when it was very active.

Table 2. *Extractive and ellagic acid contents of* Eucalyptus gigantea

Results are expressed as percentages from oven-dry wood.

Sample	Log 1			Log 2			Log 3		
	Extractive	Ellagic acid		Extractive	Ellagic acid		Extractive	Ellagic acid	
		Free	Combined		Free	Combined		Free	Combined
Phloem	—	—	—	20·1	0·36	1·26	—	—	—
Sapwood	—	—	—	10·7	0·042	0·90	3·1	0·017	0·38
Outer heartwood	11·7	1·10	2·77	18·3	1·50	3·26	6·1	0·23	0·98
Inner heartwood	6·3	0·71	1·48	10·1	0·78	1·05	—	—	—

Ether-soluble fractions. Gallic acid was the major single component (no. 10, Fig. 1, Table 3). A chromatographic estimation (spot size and intensity of colour with colour reagent (*d*) Hillis & Carle, 1959) indicated that 0·17 and 0·08 % of gallic acid was present in these samples of outer and inner heartwood respectively.

Ethyl acetate-soluble fractions. Components 18–28 (Fig. 1, Table 3) were present. In view of the hydrolytic products and the absence of acidic properties in the components, it is evident that the chebulinic acids, the chebulagic acids and chebulic acid were absent. This view was supported by comparison with the R_F values of the components when two-way chromatograms were prepared with the solvents of Hathway (1956) and White (1956). The properties of components 18–21, 23, 24 and 26–28 suggest that they are isomers of corilagin and juglanin. The chemistry of some of these components is being studied.

Insoluble material. Almost pure ellagic acid separated from the methanol extracts during extraction with other solvents. The other substance present appeared to be identical with a material found in the pipes of pulp digesters when *E. gigantea* is processed, and which had the properties of either a polymer of ellagic acid or a complex of it with some other substance (Hillis & Carle, 1959).

Residual methanol extracts. These materials were immobile in solvent 2, formed a dark-brown streak along the solvent 1 axis and became weak red (stronger at R_F 0) when sprayed with vanillin–HCl. This colour reaction indicates that some of the materials present possess a phloroglucinol nucleus with a vacant position *para* to a free hydroxyl group (Hillis & Urbach, 1959). Some of these materials could be either polymerized catechin or leucoanthocyanin. The red produced when they were heated with butanol–HCl indicated that flavandiol nuclei were present. Chromatography (in solvent 3) of the product suggested that cyanidin and delphinidin were formed but the spots were not discrete. Other products present are probably similar to the oxidation products of tea (Roberts, 1958), as leucodelphinidin could be oxidized in the presence of gallic acid to form tropolones in a manner analogous to that of gallocatechin. When the black residual methanol extracts were hydrolysed the amounts of ellagic acid produced were greater than that expected from the traces of components 18, 20 and 24 still present. The ellagic acid could arise from the oxidation products of these components or from ellagitannins which were immobile in solvent 2.

Extracted wood. When the completely methanol-extracted ground wood was extracted with warm 2N-NaOH a further amount of ellagic acid was removed (0·035 % of the outer heartwood, Hillis & Carle, 1959). This ellagic acid was probably in the cell wall and other evidence (D. E. Bland, personal communication) exists which supports this view. Also, when this wood was heated with butanol–HCl, a reddish-brown solution was obtained but the wood became red. Even after the solution was purified a red streak was obtained on the chromatograms (solvents 3 and 7) although it appeared to be strongest at the R_F of delphinidin. The colour of the treated wood could not be removed with alcohol, indicating that the substance containing the flavandiol nucleus (or the red-forming material) was incorporated into the cell wall. As only a very faint red colour was formed when the extracted wood was treated with vanillin–HCl, the phloroglucinol nucleus of the leucodelphinidin (if present) must be linked through the C_8 position to lignin or to the cell wall.

General. When samples of the petroleum ether and ethyl acetate fractions were heated with butanol–HCl, either no colour or a faint brown was obtained. Therefore the materials contained in these fractions do not interfere with the butanol–HCl staining technique (Hillis, 1958).

Chromatographic comparison of phloem and woody tissues of Eucalyptus gigantea *and* Eucalyptus sieberiana

When two-dimensional chromatograms (solvents 1 and 2) of the extracts of phloem, sapwood and heartwood were prepared with the same spot area of ellagic acid and compared, the following observations were made. (The compounds were identified

Table 3. *Major phenolic substances of* Eucalyptus gigantea *and* Eucalyptus sieberiana

+, Present; tr, trace; a, absent.

Component	No. on Fig. 1	$100 \times R_F$ (solvents 1 and 2)	Material examined: *E. gigantea* Heartwood Inner	*E. gigantea* Heartwood Outer	*E. gigantea* Sapwood	*E. gigantea* Phloem	*E. gigantea* Kino	*E. sieberiana* Sapwood	*E. sieberiana* Phloem	*E. sieberiana* Kino
Lignan (?)*	1	00/97	+	+	a	a	a	a	a	a
Unidentified	2–5	—	tr†	tr†	a	a	a	a	a	a
Unidentified	6	60/80	+†	+†	a	a	a	tr	a	a
Unidentified	7–9	—	tr†	tr†	a	a	a	a	a	a
Gallic acid	10	40/59	+	+	tr	tr	a	+	tr	a
(+)-Catechin	11	40/59	a	a	+	+	a	+	+	a
Unidentified	12	50/46	tr	tr	a	a	a	+	+	a
Unidentified	13	74/53	a	+‡	a	a	a	a	a	a
(−)-*epi*Catechin	—	38/41	a	a	a	a	a	tr	tr	a
Ellagic acid	14	00/35	+	+	tr	+	tr	+	+	tr
Unidentified	15	01/23	+	+	+	+	a	+	+	a
Unidentified	16	26/31	+	+	a	a	a	a	a	a
Unidentified	17	22/26	+	+	a	a	a	a	a	a
Ellagitannins;										
Unidentified	18	19/8	+	+	+	tr	a	+	+	a
Unidentified	19	22/18	+	+	+	+	a	tr	tr	a
Unidentified	20	32/05	+	+	+	+	a	+	+	a
Unidentified	21	36/15	+	+	+	+	a	tr	tr	a
Corilagin	22	39/20	tr	tr	tr	tr	a	tr	+	a
Unidentified	23	45/25	+	+	+	+	a	+	+	a
Unidentified	24	47/06	+	+	+	+	a	+	+	a
Juglanin	25	50/14	+	+	+	+	a	+	+	a
Unidentified	26	55/20	+	+	+	+	a	+	+	a
Unidentified	27	66/10	+	+	+	+	a	+	+	a
Unidentified	28	66/14	+	+	+	+	a	+	+	a
1-Galloyl-β-glucose	—	68/27	a	a	a	a	a	a	+	a
1:3:6-Trigalloylglucose	—	12/32	a	a	a	a	a	a	a	a
Shikimic acid	—	42/77	a	a	a	a	a	a	+	a
Leucodelphinidin	—	45/20	a	a	a	a	tr	a	a	tr

* For properties see Hillis & Carle (1958); alkoxyl groups (Zeisel, 1885) present, lignin absent.
† Much smaller amounts were in logs 2 and 3.
‡ Absent in logs 2 and 3.

by two-dimensional cochromatography and colour reactions.)

Eucalyptus gigantea. The ellagitannins (18–28) and ellagic acid (14) were the main components resolved (see Table 3). The phloem and sapwood contained more ellagitannins in relation to ellagic acid than did the heartwood (see also Table 1). The ratio of the amounts of components 18–28 to each other was about the same in all tissues. Polymerized leucoanthocyanin was present in all tissues.

Eucalyptus sieberiana. The composition (Table 3) of the extracts from the phloem of a 5-year-old tree (log 5) closely resembled that of cambium from other trees of the same species (Hillis & Carle, 1958). The phloem contained a higher ratio of resolved ellagitannins to unresolved material than did the sapwood, in particular β-glucogallin (1-galloyl-β-glucose; Fischer & Bergmann, 1918), juglanin and component 24. The phloem resembled sapwood in that it contained larger amounts of free ellagic acid than did cambium.

The heartwood from a 100-year-old specimen (log 4) did not contain (+)-catechin, in contrast with that from a younger tree (about 25 years old, Hillis & Carle, 1958).

Examination of kino

When a sample of *E. sieberiana* kino, which had been exuded in the preceding 24 hr., was chromatographed only very small traces of substances were resolved (see Table 3). The streaks which formed on the axes of solvents 1 and 2 gave deep blue with reagent (*d*) and an intense red with reagent (*f*); they also had a light-blue ultraviolet fluorescence when fumed with ammonia. The kino of *E. gigantea* behaved identically.

Delphinidin and traces of cyanidin were produced when the kinos of *E. sieberiana* and *E. gigantea* were heated with butanol–HCl. On analysis (Swain & Hillis, 1959) the kino of *E. sieberiana* gave an absorption at 560 mμ equivalent to a production of 2·8 % delphinidin. This low yield indicates that some oxidative polymerization involving direct linkage between aromatic nuclei has taken place. After acid-treatment, 9 % (w/w) of ether-soluble material was obtained from *E. sieberiana*. This material contained large amounts of phloroglucinol and gallic acid, traces of ellagic acid (less than 1 %) and four other substances, but sugars were absent. The amount of ellagic acid present was greater than that in the untreated kino. Acid-treated *E. gigantea* kino gave an identical two-way chromatogram.

DISCUSSION

Kino. The kinos of *E. sieberiana* and *E. gigantea* could have been formed *in situ* or originated elsewhere. If the kino had been translocated, evidence of a discoloured zone around the kino vein would be expected. However, the phloem adjacent to the freshly formed kino vein of *E. sieberiana* was identical to that diametrically opposite, when examined visually or when the extractives were examined chromatographically. Examination of kino veins in woody tissue (Hillis, 1958) also showed that there was no concentration of leucoanthocyanin in the rays of the woody tissue leading to the vein. The technique used in that study showed that only a few of the rows of cells surrounding the vein contained large amounts of leucoanthocyanins and this suggests that the leucoanthocyanins and the kino are formed *in situ*. In addition the kinos yielded on acid treatment some components which were not observed in the hydrolysed phloem and woody tissues; if translocation of kino had occurred these components would be found in those tissues.

The kino could be formed *in situ* from translocated shikimic acid, the contents of the cambial cells in the injured region or from translocated carbohydrate. Although shikimic acid has been found in the cambium (Hillis & Carle, 1958), this acid was absent in freshly exuded *E. siberiana* kino. As the volume occupied by the cambial cells is usually less than that of the kino (particularly in the kino pockets), it is unlikely that the cambial cells can provide all the material necessary for the formation of kino. The recent investigations of Zimmermann (1957*a*, *b*) showed that the sieve tubes of the phloem of several species (of genera other than *Eucalyptus*) contain a solution consisting almost entirely of sugars of the raffinose family and mannitol, and that polyphenols were present in only very small amounts. This evidence suggests that kino is formed *in situ* from translocated sugars (light is not essential for the formation of the C_{15} nucleus; Kursanov & Zaprometov, 1958). This view is supported by the observation that kino did not begin to exude from the injuries until the epicormic shoots had a number of leaves which could synthesize sugars. The epicormic shoots possessed chlorotic leaves as did the injured branches of *Myoporum platycarpum*, which exuded mannitol from the cambial region (Hatt & Hillis, 1947).

Cambium. The amount of leucoanthocyanins in the cambium is high during the period of its greatest activity (Pigman, Anderson, Fischer, Buchanan & Browning, 1953; Hillis, 1956). The decrease in amount with decrease in activity of the cambium could be due to translocation of leucoanthocyanins to other tissues, although there is evidence against this view (Hillis, 1956; Hathway, 1958). In view of the association between the presence of leucoanthocyanins and the process of

lignification in many plants (Bate-Smith & Lerner, 1954; Hillis & Swain, 1957), it would seem that the decrease of leucoanthocyanins in the cambium could be due to conversion into lignin, either by combination with other lignin precursors or by methylation. In support of the latter view other work has shown that the methoxyl content of Klason 'lignin' in the cambium and developing sapwood is less than that of true lignin (Stewart, Amos & Harvey, 1953), and that both the 'lignin' and the methoxyl content of it increase as the growing season progresses (Hillis & Carle, 1958). It is not possible with these species to determine whether the leucoanthocyanin has combined with the cellulose independently of lignin.

In addition to polymerized leucoanthocyanins, the major components in active *E. sieberiana* cambium have been shown to be catechin, *epi*-catechin, shikimic acid and ellagic acid–gallic acid–glucose derivatives ('ellagitannins'); the latter were the most important components (Hillis & Carle, 1958).

Phloem and sapwood. The significant differences between the constituents of phloem and sapwood and those of cambium were the increase of free ellagic acid, the presence of gallic acid, a pronounced increase of unresolvable material and the relative decrease of ellagitannins. Hathway (1957) has suggested that free ellagic acid may be formed, under certain conditions, from glucogallin and ellagitannins (see also Schmidt, 1954). Similarly this work suggests that during metabolism in the ray parenchyma cells of the phloem and sapwood the free ellagic acid was formed from the glucosides (ellagitannins) and not by direct synthesis. The glucosides could be formed in the cambial cells and remain there during transformation into phloem or sapwood cells, and in addition be formed *in situ*. As expected from the work of Roberts (1957), the gallic acid was oxidized to unidentifiable materials before the catechin, which was still present.

Heartwood. In view of the low solubility of ellagic acid it is unlikely that significant quantities of this compound could be translocated to the heartwood periphery. The free ellagic acid in this region is probably formed as a result of hydrolysis of ellagitannins in the cells, at the time of the intense metabolism (Chattaway, 1952) preceding the death of the living horizontal and vertical parenchyma cells of the sapwood when heartwood is formed. King & White (1957) have also reported that the galloylglucose derivatives in the sapwood cells of quebracho disappeared at the periphery of the heartwood. The amount of combined ellagic acid in the sapwood could give rise to the free ellagic acid in the heartwood but the amount of combined ellagic acid in the latter region has also increased (Table 2). The ratio of free ellagic acid to combined ellagic acid is higher in the heartwood than in the sapwood, confirming that there is a region of greater metabolism at the heartwood periphery.

By means of studies involving a staining technique for leucoanthocyanins, it was found (Hillis, 1958) that the amount of leucoanthocyanins in *E. camaldulensis* increased gradually from the cambium through the sapwood to the sapwood–heartwood boundary. At the boundary, the amount of leucoanthocyanins suddenly increased in the horizontal and then the vertical parenchyma cells. This analogy suggests that the increase in combined ellagic acid in the heartwood of *E. gigantea* took place at the heartwood periphery.

The amounts of ellagic acid and extractives in the inner heartwood were smaller than those in the outer heartwood (Table 1). The ratio of free to combined ellagic acid in the inner heartwood is a little higher than the ratio in the outer heartwood, so that apparently some change in the ellagitannins takes place as the heartwood of *E. gigantea* ages. It has also been observed that the amounts of catechin and monomeric leucoanthocyanins decrease from the heartwood boundary to the centre of the tree (Hillis, 1956; King & White, 1957; Hasegawa, 1958; Roux, 1958; Roux & Evelyn, 1958). Occasionally these changes are delayed (Hillis, 1956), which enabled the detection of catechin in the heartwood of a young *E. sieberiana* tree (Hillis & Carle, 1958), whereas it was absent in the heartwood of a larger tree.

General. The above data support the view that leucoanthocyanins (Bate-Smith & Metcalfe, 1957; Hillis & Swain, 1959) and other phenolic substances (Hillis & Carle, 1958) are formed within the living cells of the cambium, sapwood and phloem. Differences in the activities of the enzyme systems in different tissues could be responsible for the differences in the composition of the phenolic constituents found in those tissues (see also Hillis & Swain, 1959). As some evidence indicates that translocation of phenolic substances does not take place to a significant extent, they must be synthesized from alicyclic acids or carbohydrates. Shikimic acid has been detected in the phloem from a young tree, cambium and newly formed sapwood of *E. sieberiana* (and in other trees, Hasegawa, Nakagawa & Yoshida, 1957), but as it can be formed in shaded *E. sieberiana* leaves (W. E. Hillis, unpublished work) the acid in the woody tissues could be formed *in situ*.

The evidence presented indicates that most of the phenolic constituents in the heartwood are formed at the heartwood boundary from the starch and glycosides stored in the sapwood cells (Hillis & Carle, 1958) and also from translocated carbohydrate.

SUMMARY

1. The distribution of phenolic substances in the woody tissues and kino of *Eucalyptus gigantea* and *E. sieberiana* is described.

2. Ellagic acid is present to a varying degree in all woody and phloem tissues of these species.

3. The ellagitannins present include juglanin, corilagin and, in the phloem of young *E. sieberiana*, β-glucogallin also. The ratio of free to combined ellagic acid is low in the sapwood, higher in the phloem and higher still in the heartwood. The amount of free and combined ellagic acid is higher in the heartwood than in the sapwood.

4. Catechin is present in the bark and sapwood of both species and in the cambium of *E. sieberiana*. Shikimic acid and β-glucogallin is present in the cambium and phloem of young *E. sieberiana*. Traces of polymerized leucodelphinidin and leucocyanidin were present in all woody tissues examined.

5. The substances that are removed from the methanol extract of heartwood with light petroleum, ether and ethyl acetate do not give a red colour when heated with butanol–hydrochloric acid.

6. The kinos from these species contain polymerized leucodelphinidin and traces of leucocyanidin and ellagic acid. On acid-treatment gallic acid and further amounts of ellagic acid are liberated, and, in addition, phloroglucinol and other substances not present in the woody tissues.

7. Present evidence indicates that kino and the phenolic constituents in woody tissues are formed *in situ* from starch, glycosides and translocated carbohydrate.

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The Effect of Vitamin B_{12} on the Metabolism of Formate and Certain Formate Precursors by the Rat

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The ability of vitamin B_{12} (cobalamin) to spare the dietary requirement of animals for labile methyl donors, such as choline or methionine, is now well established (for a review see Arnstein, 1958*a*). This effect appears to be due to stimulation of synthesis of methyl groups, as shown by an increased utilization of a variety of ^{14}C-labelled precursors, including formate (Arnstein & Neuberger, 1953), methanol (Verly & Cathey, 1955), the α-carbon atom of glycine (Stekol, Weiss & Weiss, 1952; Arnstein & Neuberger, 1953) and the β-carbon atom of serine (Arnstein & Neuberger, 1953). It has also been known for some time that certain micro-organisms, notably *Escherichia coli*, require vitamin B_{12} for the biosynthesis of the methyl group of methionine (Davis & Mingioli, 1950), and considerable progress has been made towards elucidating the biochemical function of the vitamin in this reaction (Guest, 1959; Woods, 1958). There is some doubt, however, to what extent this function of vitamin B_{12} in the biosynthesis of labile methyl groups can account for its physiological importance, particularly in animal metabolism where methionine is an essential amino acid which can only reduce the requirement for vitamin B_{12} but not replace it completely.

In order to investigate this problem and the specificity of the action of vitamin B_{12} on biosynthesis of methyl groups, a comparison has now been made of the effect of vitamin B_{12} on the conversion of formate and two formate precursors, serine and histidine, into methyl groups and on the metabolism of formate and histidine into other products such as serine. Some of the results of this work have been published in a preliminary note (Arnstein, 1958*b*).

EXPERIMENTAL

Animals and diets. Albino rats of Institute stock were used, most experiments being done with litter mates (see Table 1). All animals were bred from mothers fed on a vitamin B_{12}-low diet (diet A) during pregnancy and until weaning. Deficient animals were then fed on the diets stated in Table 1, without vitamin B_{12}, and control animals were given the same diet with vitamin B_{12} at various times after weaning as described later.

Diet A had the composition (g./100 g. of diet): maize starch, 39; sucrose, 36; ethanol-extracted casein, 12; arachis oil, 6·4; cod-liver oil, 1·6; salt mixture (Glaxo Laboratories Ltd. no. DL 6), 4; L-cystine, 0·35; DL-threonine (omitted after 80 g. body wt. was reached), 0·1; succinylsulphathiazole, 0·5. Vitamins were added in the amounts (mg./kg. of diet): thiamine, 10; riboflavin, 10; pyridoxine, 10; nicotinic acid, 10; *p*-aminobenzoic acid, 10; calcium D-pantothenate, 50; inositol, 100; biotin, 0·1; pteroylglutamic acid, 2; α-tocopherol, 40; vitamin K, 1; choline chloride, 2500.

These modified diets have also been used (see Table 1): Diet A/L: identical with diet A, except that lactose replaced sucrose. Diet B: identical with diet A, except that cod-liver oil was replaced by arachis oil with vitamin A (8000 i.u./kg. of diet) and vitamin D_2 (4000 i.u./kg. of diet). Diet B/A: identical with diet B, except that succinylsulphathiazole was omitted and aureomycin (200 mg./kg. of diet) was added 3 weeks after weaning. Diet C (g./100 g.): soya-bean meal, 36; sucrose, 25; starch, 26; arachis oil, 8·; salt mixture, 4; DL-methionine 0·6; succinylsulphathiazole, 0·5; vitamins as in diet B.

The extracted casein was prepared by boiling vitamin-low casein (Genatosan Ltd., Loughborough) six times with ethanol (4 vol.). This procedure decreased the vitamin B_{12} content from about 8 to less than 1 (usually 0·6) μmg./g. of protein, as estimated by microbiological assay with *Ochromonas malhamensis* and *Lactobacillus leichmannii*, kindly carried out by Dr J. E. Ford, National Institute for Research in Dairying.

Food consumption and body weight. All animals were weighed three times a week. The food consumption of deficient rats was almost the same as that of the control animals. Details of changes in body weight and of the food consumption during the experimental feeding period are given in Table 1.

Administration of labelled compounds. All labelled compounds (obtained from The Radiochemical Centre, Amersham, Bucks.) were diluted with carrier to give the following specific radioactivites, and the diluted substances were added to the diet: (*a*) Sodium [^{14}C]formate, 0·56 μc/mg. (expt. 1, 1·12 μc/10 g. of diet), 0·78 μc/mg. (expt. 2, 1·56 μc/10 g. of diet), 0·74 μc/mg. (expts. 3 and 4, 1·46 μc/10 g. of diet); (*b*) L-[2 ring-^{14}C]histidine monohydrochloride monohydrate, 0·48 μc/mg. (0·48 μc/10 g. of diet); (*c*) DL-[3-^{14}C]serine, 0·816 μc/mg. (0·816 μc/10 g. of diet). In Expt. 5, sodium [^{14}C]formate (0·73 μc/mg.) was injected intraperitoneally, 5·83 μc (8 mg.) being used for rats 63/1 and 63/2 and 2·92 μc (4 mg.) for rats 63/4 and 63/5.

Administration of vitamin B_{12}. Cyanocobalamin (Cytamen, Glaxo Laboratories Ltd., Greenford) was administered to control animals in the amounts shown in Table 1. In Expts. 1 and 2 it was given orally from weaning until the animals were killed, i.e. for 31 and 58 days respectively. In the other experiments it was injected intraperitoneally

twice weekly in equal doses, starting 17 days (Expts. 3 and 4), 12 days (Expt. 6, litter 54), 18 days (Expt. 6, litter 55) or 23 days (Expt. 7) before the end of the experiment. In Expt. 5, rat 2 was given 10 μg. on the last and fifth day and rat 5 was given 10 μg. on the last, ninth and thirteenth day before the injection of the labelled formate.

Vitamin B_{12} content of tissues. Vitamin B_{12} was estimated microbiologically with *Ochromonas malhamensis* as test organism, essentially by the method described by Ford (1953).

Isolation of metabolic products

Protein and amino acids. The animals were killed by dislocation of the spine and the viscera (liver, kidneys, intestine, spleen, heart and lungs) were quickly removed, washed with water and disintegrated in cold ethanol in an Ato-Mix blender. The tissue was extracted six times with boiling ethanol–ether (3:1, v/v) and finally with ether. The dry, defatted tissue was extracted three times with 20 vol. of aq. 10% NaCl at 100° for 24 hr. to remove nucleic acids. The residue was washed twice with water, ethanol and ether and will be referred to as protein.

The protein (approx. 4 g.) was hydrolysed in a sealed tube with 5N-HCl (10 vol.) at 105° for 24 hr. The cooled solutions were diluted with water, filtered and evaporated to dryness *in vacuo*, the last traces of HCl being removed in a vacuum desiccator over KOH. Amino acids were separated by chromatography, first on an anion-exchange resin (De-Acidite FF, The Permutit Co. Ltd., London) in the acetate form to separate the acidic amino acids, followed by a cation-exchange resin (Zeo-Karb 225, The Permutit Co. Ltd., London) for the neutral and basic amino acids, essentially as described by Hirs, Moore & Stein (1954), but with 1·5N- and 2·5N-HCl as eluent for the cation-exchange-resin column (Stein & Moore, 1949). About 100 g. of De-Acidite resin and 300 g. of Zeo-Karb 225 resin in columns of 2·5 cm. and 3 cm. diameter respectively were sufficient for the preliminary separation of the required amino acids.

Glutamic acid and aspartic acid were isolated from fractions containing the pure amino acids by evaporating the solution to dryness, removing excess of HCl in a vacuum desiccator, dissolving the residue in a little water and adding pyridine to pH 5·0. If necessary, ethanol was added to complete the crystallization. The amino acids were recrystallized from water by addition of ethanol. There was no change in their specific radioactivity on further recrystallization. Histidine was converted into the monohydrochloride monohydrate by a similar procedure.

The other amino acid hydrochlorides were converted into the free amino acids by adsorption on Zeo-Karb 215 cation-exchange resin (The Permutit Co. Ltd., London) and displacement with aq. 0·02N-NH_3 soln. The eluate was evaporated to dryness and the residue was crystallized from aq. ethanol. Serine was usually contaminated by threonine and was purified by conversion into the hydroxyazobenzenesulphonate, which was recrystallized two to three times from water.

In Expts. 3 and 6*b*, methionine was separated from leucine by chromatography on Whatman no. 3MM paper with pentan-1-ol–pyridine–water (7:7:6, by vol.). This procedure gave pure methionine in the first case, but in Expt. 6*b* the two samples still contained 10 and 15% of leucine respectively (estimated by quantitative paper chromatography). Since leucine was, however, non-radioactive, the specific radioactivity of the methionine (Table 3) has been corrected for this inactive impurity. In Expt. 1, methionine was oxidized to the sulphone with bromine–water and separated from leucine on a column of Dowex-50 cation-exchange resin (8% cross-linked, Microchemical Specialities Inc., Berkeley, Calif., U.S.A.) (1·5 cm. diam. × 55 cm.) with 1·5N-HCl as eluent. Methionine sulphone was eluted first. The appropriate fractions were evaporated to dryness and the free amino acid was obtained by displacement from Zeo-Karb 215 resin with dilute aq. NH_3 soln. as described above. Methionine sulphone was recrystallized from water by addition of ethanol.

The purity of all amino acids isolated was checked by paper chromatography, with phenol–water (5:2, v/v, with NH_3 in the tank) and pentan-1-ol–pyridine–water (7:7:6, by vol.).

Choline. Choline was isolated by the method of du Vigneaud, Cohn, Chandler, Schenk & Simmonds (1941). The conversion of choline reineckate into chloroplatinate was carried out either as described or, more conveniently, by the following procedure. Choline reineckate was dissolved in aq. 50% acetone and the solution was passed through a column of Zeo-Karb 225 (H^+ form) which had been washed free of excess of acid. The column was washed with aq. 50% acetone until the effluent was colourless, then with a little water. Choline was eluted with 2N-HCl and the solution was evaporated to dryness. The choline chloride was converted first into the mercurichloride complex (m.p. 170°) and finally into the chloroplatinate, as previously described (Arnstein & Neuberger, 1953). The chloroplatinate was recrystallized from water by addition of ethanol, the specific radioactivity remaining unchanged. The purity of the salt was checked by Pt analysis, the found values being within 0·4 of the theoretical value in all cases.

Choline was degraded into trimethylamine by the method of du Vigneaud *et al.* (1941), the purity of the trimethylamine chloroplatinate being checked by Pt analysis (values found were within 0·4 of the theoretical value). In Expt. 7 (deficient group), however, the sample was contaminated with ammonium chloroplatinate (see Arnstein, 1952). This sample was therefore also analysed for carbon by microanalysis and its reported specific radioactivity has been corrected for the low carbon content (Found: C, 6·87. Calc. for $C_5H_{20}N_2Cl_6Pt$: C, 13·6%).

Oxidation and excretion of formate (Expt. 5). After injection of the labelled formate (see above) the animals were placed in desiccators, through which CO_2-free air was passed slowly. The expired CO_2 was trapped in excess of 40% NaOH and converted into $BaCO_3$ by addition of $Ba(OH)_2$. The $BaCO_3$ was washed with water, ethanol and ether, dried, weighed and counted. In the experiment with the first pair of rats the NaOH was changed at 2 and 4 hr., in that with the second pair at 1, 2, 3, 4, 6, 8, 10 and 12 hr. after the injection of the formate, CO_2 being collected for a further 2 and 13 hr. respectively.

Urine was collected either separately for the first 6 hr. and the subsequent 18 hr. (rats 1 and 2) or for 24 hr. (rats 4 and 5). It was stored at −20°. Carrier sodium formate (27·5 mg. to the 6–24 hr. collection, 68·7 mg. to the other samples) was added, the formate was oxidized to CO_2 as described by Weinhouse & Friedmann (1952) and counted as $BaCO_3$.

Radioactivity measurements

^{14}C was estimated by counting 'infinitely thick' samples on 1 or 2 cm.^{2} polythene disks (Popják, 1950), with an end-window Geiger–Müller tube and Dekatron scaler. A 1 cm.^{2} disk of poly-[^{14}C]methyl methacrylate (The Radiochemical Centre, Amersham, Bucks.), specific radioactivity 1 μc/g., was used as a reference standard. The standard error of all counts is less than 5%.

RESULTS

Effect of vitamin B_{12} on the growth and food consumption of deficient rats and on the vitamin B_{12} content of their internal organs

With all casein diets there was a high mortality of some litters within a few days of birth, but survivors grew well and the administration of large

Table 1. *Food consumption, growth and liver vitamin B_{12} content of deficient and control rats*

Compositions of the diets and other experimental details are given in the text. Ages of the animals (column 4) and the initial body weights (column 7) refer to the beginning of the administration of labelled compounds, the increase being the gain in weight during the subsequent 7 days. In Expt. 5, however, the body weights are those before fasting and the animals were killed 24 hr. after injection of labelled formate.

Expt.	Litter and rat no.	Sex	Age (days)	Diet	Amount of vitamin B_{12} given	Body wt. (g.) Initial	Body wt. (g.) Increase	Food consumption (g./7 days)	Vitamin B_{12} content of liver (μmg./g.)
					[^{14}C]Formate experiments				
1	50/1	M.	50	A	1 μg./day	144	30	102·4	29
	50/8	F.	50	A	1 μg./day	114	15	80·8	67
	50/2	M.	50	A	None	146	29	106·8	7·8
	50/4	F.	50	A	None	117	10	79·3	9·1
2*a*	52/1	M.	76	A/L	2·5 μg./day	224	18	97·5	138
	52/2	M.	76	A/L	2·5 μg./day	234	12	105·0	112
	52/4	M.	76	A/L	None	217	2	99·8	9·0
	52/5	M.	76	A/L	None	230	5	104·1	10
2*b*	52/3	F.	76	A/L	2·5 μg./day	155	2	80·6	150
	52/6	F.	76	A/L	None	163	5	80·8	52*
3*a*	57/5	M.	50	C	20 μg./week	129	20	106·2	102
	57/1	F.	50	C	20 μg./week	115	8	70·5	119
	57/6	M.	50	C	None	142	23	113·2	18
	57/2	F.	50	C	None	106	14	75·7	91*
3*b*	57/7	M.	50	B	20 μg./week	178	38	116·2	102
	57/3	F.	50	B	20 μg./week	141	18	89·2	102
	57/8	M.	50	B	None	172	33	110·0	6·0
	57/4	F.	50	B	None	123	11	71·3	6·8
4	59/3	M.	47	B	20 μg./week	146	35	102·7	75
	59/4	M.	47	B	20 μg./week	142	30	94·4	58
	59/6	M.	47	B	20 μg./week	156	36	113·1	97
	59/5	M.	47	B	None	149	29	96·4	9·1
	59/7	M.	47	B	None	142	34	109·3	10·0
	59/8	M.	47	B	None	137	33	100·8	5·0
5	63/2	M.	49	B/A	20 μg.	129	—	—	45
	63/1	M.	49	B/A	None	135	—	—	8·3
	63/5	M.	57	B/A	30 μg.	176	—	—	115
	63/4	M.	57	B/A	None	176	—	—	21
					L-[2 ring-^{14}C]Histidine experiments				
6*a*	54/4	M.	48	B	20 μg./week	156	35	105·8	68
	55/2	M.	48	B	20 μg./week	138	20	81·8	105
	54/5	M.	48	B	None	158	40	114·9	12
	55/3	M.	48	B	None	160	29	99·2	18
6*b*	54/2	F.	48	B	20 μg./week	130	13	70·9	110
	54/1	F.	48	B	None	134	15	74·3	24
					DL-[3-^{14}C]Serine experiment				
7	60/3	M.	47	B	20 μg./week	142	25	110·2	125
	60/5	M.	47	B	20 μg./week	145	28	113·2	136
	60/7	M.	47	B	20 μg./week	151	30	115·4	150
	60/4	M.	47	B	None	134	14	80·7	8·8
	60/6	M.	47	B	None	145	30	108·4	2·7
	60/8	M.	47	B	None	141	31	113·1	6·8

* Unusually high values, possibly resulting from intestinal biosynthesis of vitamin B_{12}.

doses of vitamin B_{12} (1–3 μg. of cyanocobalamin/day) had little effect on growth and food consumption (Table 1). Nevertheless, the concentration of vitamin B_{12} in the liver of deficient animals was much lower than normal (Table 1), except in two cases (indicated by an asterisk) where synthesis of vitamin B_{12} by intestinal micro-organisms may have occurred. Other tissues (spleen, kidneys, heart, lung and brain) of deficient rats apparently also contained less vitamin B_{12} than those of rats fed on the same diet and given vitamin B_{12} or kept on a stock diet, but, except for brain and spleen, tissues of only one deficient animal and one animal on stock diet were examined. The values for brain and spleen (see Table 1 for details of diets and animals) were (μmg./g. of fresh tissue): deficient spleen, 5·2 (rat 63/1), 9·0 (rat 63/4), 16·1 (rat 50/3, a male litter-mate of the animals used for Expt. 1; body wt. 183 g.); normal spleen, 19·5 (rat 63/2), 55·6 (rat 63/5), 19·3 (male rat on stock diet, body wt. 178 g.); deficient brain, 2·8 (rat 63/1), 5·3 (rat 53/4), 2·6 (rat 50/3); normal brain, 13·1 (rat 63/2), 23·0 (rat 63/5); 22·3 (rat on stock diet).

Incorporation of labelled compounds into protein

The incorporation of labelled serine or histidine into the protein of mixed viscera was not increased significantly by vitamin B_{12} (Table 2). These results were confirmed by the observation that the specific radioactivity of histidine, which was isolated from the protein, was actually slightly decreased (Table 3). On the other hand, the incorporation of formate into protein was almost doubled by vitamin B_{12} (Table 2). The specific radioactivities of both serine and methionine isolated from the protein were increased to a similar

Table 2. *Incorporation of labelled serine, histidine and formate into protein*

Labelled compounds were fed for 7 days; where more than one animal was used, the average amount of isotope per rat is given. For other experimental details see text and Table 1.

Expt.	Litter and rat no.	Precursor fed: Compound	Precursor fed: μc/rat	Vitamin B_{12} given	Specific radioactivity of protein (mμc/g.)
7	60/3, 5, 7	DL-[3-^{14}C]Serine	4·58	+	40·9
	60/4, 6, 8	DL-[3-^{14}C]Serine	4·11	–	42·5
6*a*	54/4	L-[2 ring-^{14}C]Histidine	5·08	+	110·8
	54/5	L-[2 ring-^{14}C]Histidine	5·52	–	116·4
	55/2	L-[2 ring-^{14}C]Histidine	3·93	+	116·0
	55/3	L-[2 ring-^{14}C]Histidine	4·76	–	108·3
6*b*	54/2	L-[2 ring-^{14}C]Histidine	3·40	+	112·3
	54/1	L-[2 ring-^{14}C]Histidine	3·47	–	118·3
1	50/1, 8	Sodium [^{14}C]formate	10·3	+	72·0
	50/2, 4	Sodium [^{14}C]formate	10·4	–	42·4
3*b*	57/3, 7	Sodium [^{14}C]formate	15·0	+	51·6
	57/4, 8	Sodium [^{14}C]formate	13·4	–	26·3

Table 3. *Distribution of radioactivity in some amino acids isolated from the protein of animals fed with labelled formate or histidine*

The amino acids were isolated from the proteins of the mixed viscera (Table 2) as described in the text.

Expt.	Labelled precursor	Amino acid isolated	Specific radioactivity (μc/mole): Vitamin B_{12} present (*a*)	Specific radioactivity (μc/mole): Vitamin B_{12} absent (*b*)	Ratio of specific radioactivities (*a*/*b*)
1	Sodium [^{14}C]Formate	Serine*	76·0	42·3	1·80
		Methionine†	84·0	52·5	1·60
		Aspartic acid	1·73	1·36	1·28
		Glutamic acid	1·91	1·60	1·19
3*b*	Sodium [^{14}C]Formate	Serine*	62·7	23·3	2·70
		Methionine	59·0	24·7	2·40
6*b*	L-[2 ring-^{14}C]Histidine	Histidine‡	757	818	0·93
		Serine*	10·9	9·59	1·13
		Methionine	3·30	3·76	0·88

* Counted as hydroxyazobenzenesulphonate. † Counted as sulphone.
‡ Counted as monohydrochloride monohydrate.

Table 4. *Incorporation of sodium* [^{14}C]*formate,* DL-[3-^{14}C]*serine and* L-[2 *ring*-^{14}C]*histidine into choline and choline methyl groups by vitamin B_{12}-deficient and control rats*

Choline was isolated and degraded to trimethylamine as described in the text. A dash signifies that the choline sample was not degraded.

Expt.	Labelled precursor	Vitamin B_{12} given	Specific radioactivity (μmc/g.) Choline chloroplatinate	Trimethylamine chloroplatinate
1	Formate	+	401	—
		−	214	—
2*a*	Formate	+	317	—
		−	144	—
2*b*	Formate	+	384	—
		−	348	—
3*a*	Formate	+	211	—
		−	153	—
3*b*	Formate	+	412	317
		−	117	112
6*a*	Histidine	+	40·1	34·6
		−	36·6	35·6
6*b*	Histidine	+	44·4	40·8
		−	42·8	51·0
7	Serine	+	171	66·3
		−	131	21·1

Table 5. *Effect of vitamin B_{12} on the incorporation of sodium* [^{14}C]*formate into the choline of liver, intestine and other internal organs*

In Expt. 4, labelled formate was fed with the diet for 7 days to groups of three rats; in Expt. 5 labelled formate was injected intraperitoneally (see text and Table 1). Choline was isolated from the various tissues as described in the text.

Expt.	Tissue	Vitamin B_{12} given	Specific radioactivity of choline chloroplatinate (μmc/g.)
4	Liver	+	642
		−	307
	Intestine	+	448
		−	199
	Other internal organs (mixed)	+	284
		−	157
5	Liver	+	608
		−	292

extent, whereas the specific radioactivity of aspartic acid and glutamic acid was much less affected (Table 3).

Conversion of labelled formate, serine and histidine into choline

The conversion of formate into choline was markedly decreased by the absence of vitamin B_{12} (Table 4), provided that the animals had sufficiently low amounts of vitamin B_{12} in their livers. In Expts. 2*b* and 3*a* the difference in the specific radioactivities of the choline from the deficient and control animals was relatively small; in both cases the liver of one of the rats in the deficient group was found to contain an exceptionally high level of vitamin B_{12} (rats 52/6 and 57/2, Table 1).

The specific radioactivities of choline isolated separately from liver, intestine and the remaining internal organs were affected to about the same extent by vitamin B_{12} deficiency (Table 5) and the effect of vitamin B_{12} on the radioactivity of liver choline was the same whether the labelled formate was injected (Expt. 5) or fed with the diet (Expt. 4).

Degradation of the choline (Table 4) showed that with labelled formate or serine as precursor vitamin B_{12} increased the labelling of choline methyl groups approximately threefold. The radioactivity of the ethanolamine moiety of choline (obtained by difference between the radioactivities of choline and trimethylamine chloroplatinates) was also greatly increased by vitamin B_{12} when labelled formate was the precursor but not when labelled serine was used.

Table 4 also shows that vitamin B_{12} deficiency appeared to have little or no effect on the utilization of labelled histidine for the biosynthesis of either total choline or choline methyl groups.

The relative importance of serine, histidine and formate as precursors of choline and choline methyl groups is shown in Table 6, in which previous results have been expressed in terms of standardized radioactivities. Serine is the best precursor of choline, which is partly due to its extensive conversion into the ethanolamine moiety.

Table 6. *Effect of vitamin B_{12} on the relative utilization of serine, histidine and formate for the biosynthesis of choline*

The results given in Table 4 have been standardized for the administration of 100 μc/100 g. body wt. (average weight during the 7-day-feeding period). With serine, allowance has been made for the fact that D-serine is not converted into choline (Arnstein, 1951). The radioactivity of the ethanolamine moiety is obtained by difference and the error of the result is calculated on the assumption of 5% counting errors for choline and trimethylamine chloroplatinates. The choline of Expt. 1 was not degraded.

Expt.	Precursor	Vitamin B_{12} given	Standard specific radioactivity (μc/mole) Choline	Trimethylamine	Ethanolamine
7	L-[3-^{14}C]Serine	+	3680	1220	2460±245
		−	2990	413	2577±170
6*a*	L-[2 ring-^{14}C]Histidine	+	869	598	171±73
		−	771	572	199±67
6*b*	L-[2 ring-^{14}C]Histidine	+	1104	876	228±99
		−	1078	1003	75±104
1	Sodium [^{14}C]formate	+	1639	—	—
		−	641	—	—
3*b*	Sodium [^{14}C]formate	+	1473	969	504±121
		−	425	347	78±38

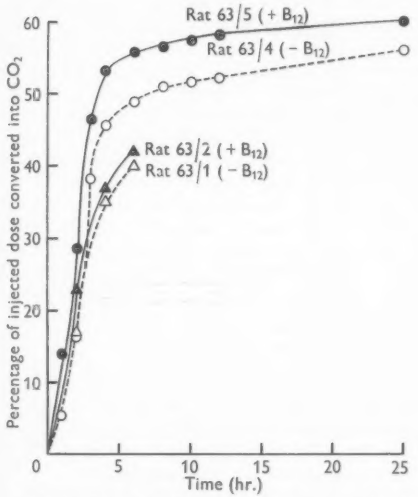

Fig. 1. Oxidation of sodium [^{14}C]formate to respiratory carbon dioxide by vitamin B_{12}-deficient and control rats. The cumulative amount of ^{14}C expired in the respiratory CO_2 during 6 and 24 hr. respectively, after injection of labelled formate, is given as the percentage of the dose. For experimental details see text.

In the presence of vitamin B_{12}, the relative efficiency of these compounds for methyl biosynthesis was serine > formate > histidine, but in vitamin B_{12}-deficient animals it was histidine > serine > formate.

Both histidine and formate are poorer precursors of the ethanolamine moiety of choline than is serine. The utilization of formate for this reaction is reduced by vitamin B_{12} deficiency, as would be expected since incorporation into serine, which is the immediate precursor of ethanolamine (Arnstein, 1951), must be an obligatory step. It should be noted, however, that the radioactivity of the ethanolamine moiety is obtained by difference and 5% errors in the counting of choline and trimethylamine could markedly affect the magnitude of the difference between the deficient and control rats. For this reason the reduction in the conversion of histidine into the ethanolamine in Expt. 6*b* may not be significant.

Oxidation and urinary excretion of formate

The effect of vitamin B_{12} deficiency on the oxidation of formate is shown in Fig. 1. The initial rate of oxidation was less in the deficient rats, but after about 2 hr. the deficient animals expired somewhat more $^{14}CO_2$ than the controls. The total oxidized in 6 or 25 hr. by the deficient animals was only slightly less than that of the controls.

The amounts of formate excreted in the urine were: rat 63/1 (deficient), 0–6 hr. 0·70 μc (12%), 6–25 hr. < 0·001 μc; rat 63/3 (control), 0–6 hr. 0·504 μc (8·6%), 6–25 hr. < 0·001 μc; rat 63/4 (deficient), 0–25 hr. 0·685 μc (23·5%); rat 63/5 (control), 0–24 hr. 0·144 μc (5·0%). Formate excretion was thus essentially complete within 6 hr. of the injection of the labelled formate and the deficient animals excreted a greater proportion of the dose than did the controls.

When these results are combined, the total amount of formate excreted and oxidized to CO_2 was: rat 63/1, 3·02 μc (51·8%); rat 63/2 (control), 2·93 μc (50·3%); rat 63/4 (deficient), 2·27 μc

(78·0%); rat 63/5 (control), 1·89 μc (65·0%). The differences between the deficient animals and the corresponding controls are thus less than 20%.

DISCUSSION

The observed stimulation of the biosynthesis of choline methyl groups from serine and formate by vitamin B_{12} is qualitatively similar to that previously obtained with rats fed on amino acid diets deficient in methionine (Arnstein & Neuberger, 1953). The greater difference in the utilization of formate by deficient and control rats in the present work is not surprising in view of the likelihood that the deficient animals were more thoroughly depleted of vitamin B_{12} since they had been specially bred from mothers also kept on a deficient diet. This interpretation is supported by the apparent correlation between the conversion of formate into choline and the vitamin B_{12} content of the liver, which must be less than about 25 μmg./g. before effects on formate metabolism can be demonstrated. The normal growth and food consumption of deficient animals suggests that the changes in formate metabolism which have been observed in this work can occur in deficiency states which are still relatively mild. These metabolic effects may thus be an early consequence of vitamin B_{12} deficiency.

Although vitamin B_{12} increased the oxidation of formate to respiratory carbon dioxide and decreased its excretion in the urine, the combined effect of these metabolic changes appears to be too small to account quantitatively for the observed increases in methyl-group biosynthesis. This conclusion is supported by the observation that vitamin B_{12} actually decreased the incorporation of formate into nucleic acid adenine and guanine (Arnstein, 1958*b*, 1959), since the radioactivity of these metabolic products should be changed similarly to that of choline if vitamin B_{12} affected mainly the oxidation and excretion of formate. It is not unlikely, moreover, that changes in the oxidation and excretion of formate could be due to a primary effect of vitamin B_{12} on formate biosynthesis from precursors such as serine, which would affect the size of the metabolic pool and hence its catabolism. That the oxidation of the hydroxymethyl group of serine to the level of formate may require vitamin B_{12} is suggested by an increased conversion of [3-^{14}C]serine into nucleic acid adenine and guanine by the vitamin (Arnstein, 1959; Arnstein & White, unpublished results). The present work shows that vitamin B_{12} also increases the reverse of this reaction, namely the reductive conversion of formate into serine. This result is noteworthy because it demonstrates that the metabolic function of vitamin B_{12} on C_1 metabolism by the rat is not restricted to methyl-group biosynthesis.

Since vitamin B_{12}, unlike folic acid, is apparently not required for the transfer of C_1 compounds at the same oxidation level, as has been shown for transmethylation (Mistry, Vadopalaite, Chang, Firth & Johnson, 1955), for the glycine–serine interconversion (Arnstein & Neuberger, 1953; Chang & Johnson, 1955; Arnstein & Stanković, 1956) and for purine synthesis from formate (Arnstein, 1958*b*), it is suggested that it functions at their interconversion by oxidation–reduction reactions. Whether the mechanism by which vitamin B_{12} stimulates the incorporation of formate into serine and methionine is similar to that involved in methionine biosynthesis from serine by *Escherichia coli* (Guest, 1959; Woods, 1958) remains to be elucidated.

It is of interest that the experiments with labelled histidine indicate that vitamin B_{12} may not be required for the utilization of this formate precursor for the biosynthesis of serine, methionine and choline. It is possible, however, that more severely deficient animals would show a requirement for these reactions. In any case, the physiological importance of vitamin B_{12} in C_1 metabolism may be due to the availability of serine, a non-essential amino acid, as a metabolic source of formate in the presence of the vitamin, whereas in its absence histidine, an essential amino acid, has to be used. One result of the relatively greater importance of histidine as a formate precursor in the vitamin B_{12}-deficient animal could be a decrease in the availability of this amino acid for other metabolic reactions, including protein synthesis.

SUMMARY

1. The metabolism of sodium [^{14}C]formate, DL-[3-^{14}C]serine and L-[2 ring-^{14}C]histidine has been studied *in vivo* in young rats depleted of vitamin B_{12} by breeding from deficient mothers which were fed on a vitamin B_{12}-low casein diet.

2. The livers of rats fed on the deficient diet contained much less vitamin B_{12} than those of control animals and other internal organs were also depleted.

3. The incorporation of serine and histidine into the mixed visceral proteins was not reduced by lack of vitamin B_{12}.

4. Administration of vitamin B_{12} to deficient rats increased the oxidation of formate to respiratory carbon dioxide and decreased the urinary excretion of formate.

5. The conversion of formate into both serine and methionine and that of serine and formate into choline methyl groups were markedly increased by vitamin B_{12}.

6. The utilization of histidine for the biosynthesis of serine, methionine and choline was not affected by the vitamin B_{12} deficiency.

7. It is suggested that vitamin B_{12} is required for the interconversion of C_1 compounds by oxidation–reduction, but not for their transfer at the same oxidation level. The possible physiological significance of this function is discussed.

I should like to thank Dr J. E. Ford for the microbiological assays of casein samples and of the livers of the first experiment and for helpful advice on the estimation of vitamin B_{12} with *Ochromonas malhamensis*. I am also grateful to Dr A. M. White for the isolation of choline in the first experiment and for advice on the use of ion-exchange chromatography in the isolation of choline and to Mrs B. Higginson for expert technical assistance throughout this work.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **388th Meeting** *of the Society was held in the Department of Physiology, Bedford College, London, on Saturday, 21 November, when the following papers were read:*

COMMUNICATIONS

Metabolism of Labelled Cerebral Lipids in Developing and Mature Rats after Injection of [1-^{14}C]Glycerol. By A. N. Davison. (*Pathology Department, Guy's Hospital Medical School, London, S.E.* 1)

After injection of radioactive isotopes into developing animals it has been found that a proportion of the radioactivity incorporated into the myelin lipids of the brain persists for at least 250 days after injection (Davison, Morgan, Wajda & Payling Wright, 1959; Davison & Dobbing, 1958, 1959*a*). This work led to the hypothesis that brain lipids can be divided into metabolically active and inactive groups. The latter are probably associated with certain anatomical structures, notably the myelin sheath. A comparison of brain phospholipid metabolism in developing and mature animals using ^{32}P has been found to be complicated by persistence of acid-soluble ^{32}P in the blood of the adults (Davison & Dobbing, 1959*b*). The comparison can be greatly simplified by using ^{14}C-glycerol, for this rapidly disappears from the blood of young and old animals.

In the present work [1-^{14}C]glycerol has been injected into 13-day-old and adult rats. The animals were killed at intervals up to 110 days after injection, brain lipids were extracted with chloroform–methanol (2 : 1, v/v) and radioactivity determined. In young animals the loss of radioactivity of total brain lipid is much slower ($t_{0.5}$ = about 135 days) than in adults ($t_{0.5}$ = about 40 days). Lipid extracts were fractionated on an alumina column into cholesterol, choline-containing phospholipids, cerebrosides and non-choline-containing phospholipids (Davison & Wajda, 1959). In the developing rats [1-^{14}C]glycerol is incorporated rapidly into the myelin lipids, sphinogomyelin, cholesterol and cerebroside, and also into cephalin and lecithin of the brain. At 110 days after injection there was little loss of radioactivity incorporated into brain cholesterol and cerebroside, although less than half the initial radioactivity was present in the sphingomyelin, cephalin and lecithin. In adult animals much less radioactive glycerol was incorporated into the myelin lipids and turnover of all the cerebral lipids labelled was more rapid. The relevance of these findings to the general hypothesis and to recent work on brain phospholipid metabolism (Davison & Dobbing, 1959*b*) will be discussed.

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The Presence of Lecithin in whole Ram Semen. By G. M. Gray.* (*Lister Institute of Preventive Medicine, London, S.W.* 1)

Lovern, Olley, Hartree & Mann (1957) concluded that the predominant lipid in ram spermatozoa was choline plasmalogen and found no evidence for the presence of lecithin. Results obtained in this laboratory, however, indicate that lecithin and choline plasmalogen are present in whole ram semen in about equal proportions.

Ram semen stored at −25° was thawed in methanol and extracted with chloroform–methanol (2:1, v/v; 19 ml./g.) at room temperature. The extract was washed three times with 1 vol. of water, dried over anhydrous Na_2SO_4 and the solvent removed *in vacuo* at 35°. The lipid was dissolved in light petroleum (b.p. 40–60°) and the non-P lipid (750 mg./100 g. of semen) removed by dialysis through a rubber membrane (van Beers, de Iongh

* Beit Memorial Fellow.

& Boldingh, 1958). The phospholipid (620 mg./100 g. of semen; P, 3·8% of dry wt.) was quantitatively recovered and the molar ratio, P:total N:amino N:fatty aldehyde:acyl ester, was 1:0·94:0·19:0·40:1·51.

The phospholipid was chromatographed on silicic acid using chloroform with increasing proportions of methanol for elution (analytical methods; Gray & Macfarlane, 1958). Two main bands *A* and *B*, corresponding to the usual cephalin and choline phosphatide fractions were obtained. Fraction *A* contained 24·8% of the total P put on the column; molar ratio, P:amino-N:aldehyde, 1:0·78:0·19. Fraction *B* contained 72% of the total P, the molar ratio, P:amino-N:choline-N:aldehyde of 1:0·03:0·92:0·50 indicating that it was an equimolar mixture of lecithin and choline plasmalogen. A small fraction following *B*, which from analysis was probably sphingomyelin, accounted for the remaining P (about 3% of total P). The presence of lecithin in fraction *B* was confirmed by treating it with 90% acetic acid at 38° overnight, in which conditions lecithin remains intact and choline plasmalogen is split to free fatty aldehyde and lysolecithin. The products were chromatographed on silicic acid with chloroform–methanol (1:1, v/v) giving a lecithin band (44% of total P applied; molar ratio, P:aldehyde:acyl ester, 1:0·02:2·05) well separated from the lysolecithin band (47% of the total P; molar ratio, P:aldehyde:acyl ester, 1:0·0:1·07).

Mann (1954) showed that 90% of the phospholipid of whole ram semen occurred in the spermatozoa, and 10% in the seminal plasma. The present results show that lecithin accounts for 35–36% of the phospholipid of whole semen and therefore must be present in the spermatozoa as a major component.

The gift of ram semen from Dr R. M. C. Dawson is gratefully acknowledged.

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Stepwise Degradation of Blood-group H Mucopolysaccharide by an Enzyme Preparation from *Trichomonas foetus*.

By H. M. Tyler and Winifred M. Watkins. (*Lister Institute of Preventive Medicine, London, S.W.* 1)

The nature of the changes induced by an enzyme in extracts of *Trichomonas foetus* which inactivates human-blood-group H-substance (Watkins, 1953, 1959) has been studied by following the progressive action of a partially purified enzyme preparation.

A crude extract from *T. foetus* was freed from enzymes which inactivate human-blood-group A-, B- and Le^a-substances by fractionation with ethanol at −6°. The resultant preparation showed enhanced H enzyme activity and a slight capacity to hydrolyse low-molecular-weight β-galactoside and β-*N*-acetylglucosaminide substrates. Human H-substance (200 mg.) was incubated for 2 hr. at 37° with this enzyme preparation (20 mg.), the mixture was dialysed to remove diffusible products, a portion of the indiffusible material was withdrawn for serological and chemical analysis and the remainder was then treated with a further 10% by weight of the same enzyme preparation. This procedure was repeated four times. During the first 2 hr. hydrolysis period the H serological activity fell to 6% of the original value, the capacity of the substance to cross-react with type XIV pneumococcus serum was increased, and 30–40% of the total fucose of the H-substance became diffusible, whereas only 1–2% of the total galactose and *N*-acetyl hexosamine were liberated. The second and subsequent enzyme treatments caused the H activity to fall still further and after 6 hr. only 16% of the original fucose remained indiffusible. Increasing amounts of galactose and *N*-acetyl hexosamine were liberated, indicating that the enzymes bringing about their release could act only after the removal of fucose. Diffusible oligosaccharides or amino acids were not detected at any stage in the hydrolysis, and examination in the ultracentrifuge of the material remaining after four successive enzyme treatments did not reveal any gross disruption of the molecules. These results are consistent with the view that the enzymes in *T. foetus* which destroy blood-group specificity act by removing terminal non-reducing sugars and that the first sugar to be removed by the H-enzyme preparation is fucose.

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Variation of Michaelis Constant and Substrate–Coenzyme Affinity with pH in Leucine Decarboxylase of *Proteus*. By H. K. KING and S. J. BROWN. (*Department of Biochemistry, The University, Liverpool, 3*)

King & Lucas (1959) reported the stability constants for the Schiff bases formed by the interaction of pyridoxal phosphate and certain amino acids, and noted that in several cases these corresponded approximately with the Michaelis constants of pyridoxal phosphate enzymes employing these amino acids as substrates. They suggested that the controlling factor in enzyme–substrate affinity in these cases was the stability of the Schiff bases formed by the coenzyme and amino acid.

We have investigated a single enzyme, the leucine decarboxylase system of *Proteus* (Ekladius, King & Sutton, 1957) at different pH values. The Schiff base stabilities were determined as before (King & Lucas, 1959) and are expressed as K, the molar concentration of leucine at which half of the pyridoxal phosphate present is combined as Schiff base. The Michaelis constants were determined on a single sample of the enzyme (prepared by disintegrating washed *Proteus* cells in the Hughes bacterial press) and the results expressed by the reciprocal plot method, which permitted deduction of both the Michaelis constants and the maximal velocity attained at complete substrate saturation. Stability constants (K) for the Schiff base (pyridoxal phosphate + leucine) were: pH 5, 0·085M; pH 6, 0·052M; pH 7, 0·027M. Michaelis constants (K_s) for the enzyme were: pH 5, 0·045M; pH 6, 0·027M; pH 7, 0·016M. The enzyme velocity at infinite substrate concentration was the same at pH 5, 6 and 7.

Over the acid range the fall in enzyme activity is thus due to weakening of enzyme–substrate affinity, and this coincides closely with the reduction in the stability of the substrate–coenzyme Schiff base.

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The Occurrence of *O*-Methyl Ethers of Rhamnose and Fucose in Specific Glycolipids of Certain Mycobacteria. By A. P. MACLENNAN,* D. W. SMITH and H. M. RANDALL (introduced by D. A. L. DAVIES). (*Department of Physics, University of Michigan, U.S.A. and Department of Medical Microbiology, University of Wisconsin, U.S.A.*)

The application of infrared spectroscopy to the characterization of mycobacteria (Smith, Randall, Gastambide-Odier & Koevoet, 1957) has led to the detection of a glycolipid (G_B) that is produced only by the bovine type of *Mycobacterium tuberculosis*, and of another (G_A) that is specific for the photochromogenic group (Runyon, 1955) of atypical mycobacteria.

In the present study G_A and G_B were isolated from alcohol–ether extracts of bacteria by chromatography on columns of alumina and of silicic acid. G_A had m.p. 109°; $[\alpha]_D^{20} - 37 \pm 1°$ in chloroform (c, 1) (Found: C, 72·6; H, 10·8; OMe, 8·5%). G_B had m.p. 23°; $[\alpha]_{20}^{D} - 26 \pm 1°$ in chloroform (c, 1) (Found: C, 77·1; H, 12·3; OMe, 4·0%). Both substances had mol.wt. of roughly 1000 (Rast) and did not contain detectable amounts of N or P.

Samples of G_A and G_B were hydrolysed with N-HCl at 100° for 16 hr. and the hydrolysates were shaken with chloroform to remove lipids. Paper chromatography of the water-soluble fractions in butanol–pyridine–water (6:4:3, by vol.) revealed 3 reducing sugars in G_A with $R_{rhamnose}$ values of 1·18, 1·28 and 1·45, whereas G_B contained only one of these, $R_{rhamnose}$ 1·28. Each of the G_A sugars was isolated by eluting bands from paper chromatograms. The free sugars reacted in a specific test for 6-deoxyhexoses (Dische & Shettles, 1948) which when applied directly to G_A and G_B showed the presence of 26 and 6%, respectively, of 6-deoxyhexose, estimated as L-rhamnose.

Demethylation with boron trichloride (Allen, Bonner, Bourne & Saville, 1958) yielded fucose from the sugar with $R_{rhamnose}$ 1·18 and rhamnose from the other two; the demethylated sugars were identified by chromatography and by paper electrophoresis in borate buffer. Comparison of the R values and other properties of the G_A sugars with those of authentic *O*-methyl sugars led to their identification as 2-*O*-methylfucose, 2-*O*-methylrhamnose (in G_B also) and 2:4-di-*O*-methylrhamnose.

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The Effect of Malonate on Respiration of Homogenates of Ox Retina. By E. Ann Burgess, Margaret Kerly and M. Ataur Rahman. (*Department of Biochemistry, University College, London, W.C.* 1)

The respiration of retina is much higher during incubation in a medium containing bicarbonate than in one buffered by phosphate (Laser, 1937; Craig & Beecher, 1943). Results of some experiments on the effect of malonate on oxygen uptake of homogenates of ox retina suggest that in the presence of bicarbonate oxidation of glucose can occur by a second pathway, supplementary to the tricarboxylic acid cycle.

Ox retina was homogenized in 0·25 M-sucrose and 1 ml. of homogenate (equivalent to 200 mg. of retina) was incubated for 30 min. at 37° in a medium containing KCl, $MgSO_4$, adenosine triphosphate, phosphate buffer and ethylenediaminetetra-acetic acid. There was some oxygen uptake without addition of substrate (31 ± 6 μl.). This was increased by addition of α-oxoglutaric acid (83 ± 7 μl.), but not by addition of glucose (27 ± 3 μl.). When $NaHCO_3$ was added to the medium (Krebs, 1950), together with substrate, there was no change in the oxygen uptake from α-oxoglutaric acid (82 ± 4 μl.) but, with glucose, uptake increased to that found in the presence of the keto acid (86 ± 7 μl.). Addition of bicarbonate alone also increased the initial rate of endogenous respiration nearly to that observed in the presence of substrate, but the rate fell before the end of the 30 min. incubation period.

When malonate was added to the incubation medium, without bicarbonate, there was a pronounced inhibition of respiration from all substrates (endogenous oxygen uptake, $6 \pm$ μl.; with added α-oxoglutaric acid, 30 ± 3 μl.; with added glucose, 16 ± 1 μl.). In contrast, when malonate was added together with bicarbonate, although oxygen uptake from α-oxoglutaric acid was still inhibited (47 ± 2 μl.), there was no inhibition of endogenous respiration (53 ± 3 μl.) and oxygen uptake from glucose was inhibited to only a small extent (63 ± 3 μl.).

Note. For most, but not all, homogenates, addition to the medium of DPN and cytochrome *c* (Futterman & Kinoshita, 1959) caused some increase in oxygen uptake from all substrates, but the addition of these cofactors did not influence the bicarbonate or malonate effects.

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Transaminations with Pyruvate in Liver of Different Animal Species. By K. Corbett and E. V. Rowsell. (*Department of Biochemistry, The University, Sheffield* 10)

Transaminations have been studied using dialysed fresh liver homogenates, with pyruvate (0·02 M), L-α-amino acids (0·02 M), and pyridoxal phosphate (10 μg./ml.) incubated at 37° under nitrogen. Transamination rates were assayed using paper-chromatographic separations, followed by reactions with ninhydrin to estimate the alanine formed (Kay, Harris & Entenman, 1956). We have been concerned to survey reactions in which pyruvate acts directly as amino acceptor. To eliminate from consideration alanine formation occurring indirectly by coupled transaminations (via glutamate), aspartate was included in each series of incubations, and positive results are reported only where alanine formation exceeded that obtained with aspartate (O'Kane & Gunsalus, 1947; Rowsell, 1956).

Rowsell & Corbett (1958) observed transaminations with L-α-amino acids and pyruvate in the livers of mouse and rat. It was noted that, as distinct from transaminations with α-oxoglutarate, only a limited number, viz. L-phenylalanine, L-methionine, L-norleucine, L-leucine, L-glutamate, L-histidine, L-arginine, L-ornithine and L-tyrosine, were active as amino donors with pyruvate. In the present survey the livers of mole, hamster and multi-mammate rat (*Mastomys coucha* or *Rattus coucha*) have been shown to promote transaminations to pyruvate with the same range of amino acids. The rates were highest in mouse then decreased in the order: multi-mammate rat, mole and hamster about the same, and lowest in the rat, i.e. an inverse correlation with their size.

With other mammals studied (guinea pig, cat, monkey, sheep, pig and calf) only L-glutamate gave rapid transamination with pyruvate. Very slow reactions with pyruvate were observed with liver homogenates in the following cases: cat, L-arginine; monkey, L-phenylalanine and L-norleucine; guinea pig, L-phenylalanine. Canary and pigeon liver pro-

moted transaminations only between ornithine and pyruvate. No transaminations with pyruvate were observed with budgerigar liver. (Glutamate was not included in the L-α-amino acids tested with birds.) The sole amphibian studied, salamander, catalysed a very slow transfer from L-phenylalanine to pyruvate and the usual rapid transfer with L-glutamate.

From the present limited survey it is possible to draw the following inferences. Where a number of L-α-amino acids are active in transaminations with pyruvate, the same pattern of specificity exists. These transaminations only occur with small mammals (which have a high metabolic rate). They are not catalysed in the livers of birds with equally high or higher metabolic rates.

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Amine Metabolism, Studied in Normal and Phenylketonuric Humans by Monoamine Oxidase Inhibition. By J. B. JEPSON,* W. LOVENBERG, PEROLA ZALTZMAN, J. A. OATES, A. SJOERDSMA and S. UDENFRIEND. (*National Heart Institute, Bethesda, Maryland, U.S.A.*)

The persistence of many of the amines produced endogeneously in man is limited by the rapidity of their subsequent oxidation. Monoamine oxidase inhibitors (MAO-I) reveal amine turnover by blocking this oxidation, thus allowing the urinary excretion of amines which accumulate in the body as a whole or possibly in the kidney only. Locally concentrated amines could be responsible for the pharmacological potentialities of these drugs (Sjoerdsma, Lovenberg, Oates, Crout & Udenfriend, 1959).

Comparisons have been made of the free amines in urine from normal humans and from patients on MAO-I therapy (usually 25 mg./day of hydrazinophenylpropane). Urinary amines were adsorbed on NH_4^+-cation exchange resin, eluted with aqueous ammonia, extracted into butanol from borate-NaCl, pH 10, and appropriately desalted and extracted into acetone for chromatography in propan-2-ol–ammonia and butanol–acetic acid systems. This technique does not allow the recovery of phenylethylamine, tryptamine, serotonin, volatile aliphatic amines, alkali-labile amines like adrenaline, nor any amino acid. Nevertheless, 20–30 ninhydrin-positive substances were obtained from normal urine, at levels estimated to be between 0·1 and 5 μmoles of each/day; some of these were considerably elevated by MAO-blockade. Amine excretion seemed independent of diet or gut flora. As well as the expected metanephrines and *p*-tyramine, the free phenolic amines of human urine found by this procedure included *o*-tyramine (only detectable on MAO-blockade, about 50 μg./day) and *m*-tyramine (just detectable normally, rising on blockade to about 100 μg./day, further increased by oral phenylalanine). Identification was by chromatographic comparison, specific chemical transformation, and enzymic oxidation.

Direct solvent extraction from alkaline urine allowed phenylethylamine to be estimated semiquantitatively by chromatography. The phenylethylamine turnover in normals has been compared to that in phenylketonurics where phenylalanine 'escape' to tyrosine is restricted. The results (μg. of free phenylethylamine/day) were: normals, less than 20; normals extensively blocked with MAO-I and fed extra L-phenylalanine, 60; phenylketonurics without drugs, 10–20; phenylketonurics blocked with MAO-I, 3000 and more. Despite the enormous excretion of *o*-hydroxyphenylacetic acid by phenylketonurics, their accumulation of *o*-tyramine was not larger than normals. Their accumulation of *m*-tyramine was less than normals, indicating a relationship between *para* and *meta* hydroxylation. Phenylethylamine is the first compound with known direct physiological activity whose turnover is greater in phenylketonurics than in normals.

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The Metabolism of 1-Bromobutane. By H. G. Bray and Sybil P. James. (*Physiology Department, Medical School, University of Birmingham*)

In a previous communication (Bray & James, 1958) evidence was presented for the formation of mercapturic acids as metabolites of 1-bromo-butane, -pentane, -hexane, -heptane and -octane. Difficulty in isolating these metabolites led us to the conclusion that all the material which reacted with nitroprusside in the method (Barnes, James & Wood, 1959) used could not be mercapturic acid. The metabolism of the 1-bromoparaffins, and particularly that of 1-bromobutane, has therefore been investigated in detail. It has been shown that at least three sulphur-containing metabolites are present in the urine of rabbits dosed with 1-bromobutane. Two of these metabolites can be extracted by ether from acidified urine and can be separated by means of paper chromatography. One of these has been obtained in crystalline form from both rabbit and rat urines, and has been shown to be *N*-acetyl-*S*-butyl-L-cysteine. The second metabolite was obtained in a fraction which was free from other sulphur-containing material and did not give a ninhydrin reaction. On hydrolysis with N-HCl the metabolite decomposed to give *S*-butyl-L-cysteine and glycine, which were identified by paper chromatography. It seems possible that this metabolite may be a derivative of a peptide of these two amino acids. The third metabolite is not extracted by ether from acidified urine and was separated from concentrated ether-extracted urine by chromatography on paper-powder columns and obtained in a fraction which gave no ninhydrin reaction and contained no other sulphur-containing material. On acid hydrolysis it yielded a number of ninhydrin-reacting substances, the chief of which were *S*-butyl-L-cysteine, glutamic acid, glycine and a sulphur-containing compound which has not yet been identified. It seems possible that this metabolite is a derivative of a more complex peptide. It does not appear to be *N*-acetyl-*S*-butyl glutathione. The urine of guinea pigs dosed with 1-bromobutane contains only the third metabolite.

Evidence has been obtained that the excretion products of the higher 1-bromoparaffins may be similarly complex. Pentyl mercapturic acid has been isolated from the urines of rabbits and rats given 1-bromopentane and hexyl mercapturic acid from that of rats dosed with 1-bromohexane.

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The Effect of 5-Fluorouracil and other Pyrimidine and Purine Analogues on Cell-wall Synthesis and the Accumulation of Amino Sugar in *Staphylococcus aureus*. By H. J. Rogers and H. R. Perkins. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

5-Fluorouracil (5-FU) (Duschinsky, Pleven & Heidelberger, 1957) is strongly inhibitory to bacterial growth (Heidelberger, *et al.* 1957), and inhibits β-galactosidase formation in *Escherichia coli* (Horowitz, Sankonen & Chargaff, 1958). When 5-fluoro[2-^{14}C]uridine is incubated with Ehrlich ascites cells, the compounds which become labelled are ribonucleic acid, uridine mono-, di- and triphosphates, and, probably, the uridine diphosphate glucose (Harbers, Chaudhuri & Heidelberger, 1959).

Uridine–amino sugar–amino acid compounds which accumulate in some strains of staphylococci when acted on by penicillin (Park & Strominger, 1957; Strominger, 1957; Ito, Ishimoto & Saito, 1958, 1959) have been suggested as biosynthetic intermediates of cell-wall mucopeptide (Park & Strominger, 1957; Strominger, 1957). Takagi & Otsugi (1958) and Otsugi & Takagi (1959) found that trichloroacetic acid-soluble *N*-acetylamino sugars accumulate in *E. coli* incubated in the presence of 6-azauracil. It was therefore of interest to examine the effects of purine and pyrimidine analogues on cell-wall synthesis in staphylococci. 6-Azathymine, 8-azaguanine, 6-azauracil and 5-FU were added to suspensions of staphylococci (strains 524 and Oxford) under conditions such that cell-wall synthesis, but no protein and little nucleic acid synthesis took place (Mandelstam & Rogers, 1958, 1959). The incorporation of [1-^{14}C]glutamate into the cell wall was inhibited from about 30 to 50 % by the presence of 0·1 μmole/ml. of 5-FU. This inhibition was reversed by uracil and uridine but not by thymine. None of the other compounds showed appreciable inhibition at similar concentrations. Examination of the accumulation of trichloroacetic acid-soluble *N*-acetylamino sugars as by Strominger (1957) showed, however, that there was a marked increase with both 6-azauracil and 5-FU, the latter compound being much the more effective. The accumulation in the presence of 5-FU was reversed to the extent

of 80% by the presence of an equimolar concentration of either uridine or uracil, but only 30–40% reversed by thymidine.

Examination of the distribution of the accumulated trichloroacetic acid-soluble *N*-acetylamino sugar compounds during fractionation on Dowex-1 columns has shown them to be almost exclusively (80–90%) contained in the two peaks showing absorption of ultraviolet light at 260 mμ, described by Strominger (1957) as containing the compounds accumulated under the influence of penicillin. The first of these peaks yielded alanine, glycine, glutamate, lysine, muramic acid and glucosamine on acid hydrolysis. The second yielded muramic acid, glucosamine, alanine, glycine and glutamate. Both peaks yielded purines and pyrimidines. Further fractionation, however, not yet completed, shows that peak 1 is complex, with more than one component containing amino acids and amino sugars.

Uracil analogues, in particular 5-FU, can then partially inhibit cell-wall synthesis in a specific manner and cause the accumulation within the cell of amino sugar–amino acid compounds, probably linked to nucleotides.

Our thanks are due to Dr Morrison of Roche Products Ltd. for a generous gift of 5-fluorouracil.

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Isolation of a Mucopolysaccharide from Cell-wall Preparations of *Bacillus subtilis*. By Ewa Janczura, H. R. Perkins and H. J. Rogers. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

In cell walls prepared from *Bacillus subtilis* (N.C.T.C. 6346) by the method of Cummins & Harris (1956), muramic acid accounted for less than half of the molecules of hexosamine present, whereas in strains of *Staphylococcus aureus* and *Micrococcus lysodeikticus* the molar ratio of muramic acid to glucosamine was about 1:1 (Mandelstam & Rogers, 1959; Perkins & Rogers, 1959). Treatment of the *B. subtilis* cell walls with 5% (w/v) trichloroacetic acid at 20° caused the excess hexosamine to pass into solution, together with other material similar to the teichoic acids described by Armstrong, Baddiley, Buchanan, Carss & Greenberg (1958). The material containing hexosamine was non-diffusible through cellophan. When subjected to paper electrophoresis at pH 2·4 it moved towards the anode, separating from teichoic acid, which moved more rapidly. Material isolated in this way was shown to contain only glucuronic acid and *N*-acetylhexosamine in equimolecular proportions. It did not contain any amino acids, sulphur or phosphorus. The hexosamine obtained by acid hydrolysis was shown to be galactosamine since (*a*) after degradation with ninhydrin it produced lyxose (Stoffyn & Jeanloz, 1954), and (*b*) its *N*-acetyl derivative ran with *N*-acetylgalactosamine on chromatography in butanol–pyridine–water (Crumpton, 1959) (i.e. it was not talosamine). Glucuronic acid was identified chromatographically both as the free acid and as the lactone. It was concluded that the material was a mucopolysaccharide which probably consisted of alternate residues of glucuronic acid and *N*-acetylgalactosamine.

The isolated mucopolysaccharide was attacked to a small extent by a commercial preparation of testicular hyaluronidase (Rondase, Evans Med. Supplies Ltd., Ruislip, Middlesex) to yield material reacting as *N*-acetylhexosamine, but it was not attacked by staphylococcal filtrates containing hyaluronidase. Like hyaluronic acid it caused a precipitate with serum reagent (Tolksdorf, McCready, McCullagh & Schwenk, 1949) or with cetylpyridinium bromide (Scott, 1955). Precipitation with the latter reagent was prevented by 0·075 M-Na_2SO_4. The isolated mucopolysaccharide had $[\alpha]_D^{22} + 40°$ (approx.) in water (*c*, 0·9). When treated with $NaIO_4$ at pH 4·5 at 5°, it used 0·55 mole per disaccharide unit (hyaluronic acid, 0·4 mole). It seems that this bacterial polysaccharide differs

from chondroitin (Davidson & Meyer, 1955) at least in being α-linked, although the points of attachment may differ too. Similar material has been found in culture filtrates of *B. subtilis*, and it may therefore be of a capsular nature.

It is proposed to call this material teichuronic acid.

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Incorporation of *p*-Fluorophenylalanine into Exopenicillinase of *Bacillus cereus* 569/H. By M. H. Richmond. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

Addition of 50 μg. of DL-*p*-fluorophenylalanine to cultures of *Bacillus cereus* 569/H (constitutive strain) growing exponentially in media lacking phenylanine led to inhibition of growth. For about one generation time after addition of the analogue growth was of exponential type but at a lower rate than that of the control; subsequently growth became approximately linear. After about 2 hr. incubation in the presence of the analogue, the differential rate of exopenicillinase formation appeared to be markedly depressed, reaching 40% of normal. Furthermore, it was possible to show that this change was associated with an increasing differential rate of incorporation of *p*-fluorophenyl[β-^{14}C]alanine into the total cell protein, the total exocellular protein and the total exopenicillinase. Investigation of the effect of the analogue on the total cell and exocellular protein showed that the *p*-fluorophenylalanine was incorporated at the expense of phenylalanine, and replaced more than 80% of the normal amino acid after 2 hr. incubation.

Study of the immunological properties of the exopenicillinase synthesized under these conditions, showed that the impaired differential rate of enzyme synthesis and the increased incorporation of the analogue were associated with the appearance of increasing amounts of material with abnormal neutralization characteristics. Normal antiserum exhibited markedly lower neutralization titres on these preparations, particularly at low antibody:enzyme ratios.

As the purification of exopenicillinase involves precipitation with antiserum (Pollock & Kramer, 1958), it was not possible to determine the specific enzyme activity of the exopenicillinase synthesized in the presence of *p*-fluorophenylalanine. However, it was found that the differential rate of incorporation into protein of any amino acid but phenylalanine was not affected by the analogue and it was therefore possible to measure the synthesis of exopenicillinase *protein* by following the incorporation of [^{14}C]valine into purified exopenicillinase. These experiments show that *p*-fluorophenylalanine had no effect on the differential rate of synthesis of penicillinase protein, but the specific enzyme activity was depressed by about 60%. In similar experiments, the incorporation of *p*-fluorophenyl[β-^{14}C]alanine was equivalent to 5–6 residues/molecule of exopenicillinase. As normal exopenicillinase contains seven phenylalanine residues, these results indicate that the enzymic and immunological properties of the exopenicillinase synthesized in the presence of *p*-fluorophenylalanine may be due to the replacement of the majority of the phenylalanine in the molecule by the *p*-fluoro derivative.

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Semi-Micro Analysis of Amino Acid Mixtures by Ion-Exchange Chromatography By J. E Eastoe. (*Department of Dental Science, Royal College of Surgeons of England, London, W.C. 2*)

The original method for quantitative amino acid analysis by elution of ion-exchange resins with buffers (Moore & Stein, 1951) required approximately 10 mg. of protein. A recent modification, suitable for use with automatic apparatus, requires only 1·5–4 mg. (Moore, Spackman & Stein, 1958). In some investigations (e.g. enamel proteins, bacterial toxins) the quantity available may be even more limited.

The method of Moore & Stein (1951) has been

modified for smaller samples by reducing the diameter of the 100 cm. and 15 cm. Dowex-50 columns from 0·90 cm. to 0·40 cm. Fractions (0·2 ml., 9 drops) were collected in 6 in. × $\frac{5}{8}$ in. test tubes and, after adjustment to pH 5·0, were heated at 100° for 20 min. with 0·4 ml. of ninhydrin reagent (Moore & Stein, 1948). The cooled solutions were diluted with 1 ml. of ethanol–water (1:1, v/v) bringing the final volume to 1·6 ml. The optical densities were measured in a standard 2 cm. cell in a 'Uvispek' spectrophotometer (Hilger and Watts, Ltd.). The cell was raised 5·3 mm. above its normal position and the exit slit height reduced by a diaphragm covering all except the lowest 4·5 mm. Adequate sensitivity was obtained using a slit width of 0·05 mm. and a control setting of 4. The modified colorimetry gave results closely agreeing with those of the ordinary method.

The method was tested with approximately 0·3 mg. loadings of collagen and gelatin hydrolysates. The resolution and individual spread of the peaks were indistinguishable from results obtained using standard 0·9 cm. columns, showing that the relative increase in area of the 'Pyrex' glass wall had little effect. The quantities of amino acids found were comparable with those using the standard method, within the limits of experimental error. The error of this method was somewhat greater than with the original method, being approximately ±5%.

Where the quantity of material is very limited, the entire analysis may be carried out with 0·3 mg., by first separating the basic amino acids on a 2·2 × 0·40 cm. Dowex-50 column, eluted with 0·1 M-citrate buffer, pH 5·0. The first 1·2 ml. of eluate are brought to pH 2 and later transferred to the 100 cm. column. The resin, containing only basic amino acids, is placed on top of the 15 cm. column.

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The Gross Chemical Composition of Subcutaneous Adipose Tissue in the Lean and Obese Human Subject. By G. L. S. Pawan and M. Clode. (*Medical Unit, Middlesex Hospital, London, W. 1*)

In many classical metabolic studies, human adipose tissue has been assumed to contain about 10% of water. More recently, various workers have assumed higher values based on the analytical data of Forbes, Cooper & Mitchell (1953), of Vague & Garrigues (1955), and others.

We considered it worthwhile to analyse specimens of subcutaneous adipose tissue taken from the same area of the abdominal wall of lean and obese persons to ascertain if any differences in gross composition were apparent. Specimens of adipose tissue were obtained by biopsy from 6 lean and 7 obese persons, and from 14 lean and 14 obese individuals within 24 hr. *post mortem*.

Biopsy adipose tissue from the lean subjects contained 18·2–50·6 (mean 28·6)% water, 61·4–82·4 (mean 68·4)% lipid, and 1·8–2·8 (mean 2·1)% protein. Biopsy adipose tissue of the obese subjects contained 12·7–30·1 (mean 18·8)% water, 67·0–85·1 (mean 78·7)% lipid, and 1·8–2·7 (mean 2·0)% protein.

Post-mortem subcutaneous adipose tissue from the lean subjects contained 9·6–53·0 (mean 26·1)% water, 42·8–85·9 (mean 72·1)% lipid, while that of the obese contained 6·7–28·9 (mean 14·6)% water, and 66·6–91·0 (mean 78·8)% lipid. The iodine values (Wijs) of the fatty acids of subcutaneous adipose tissue in the lean were 71·1–74·8 (mean 73·7) and in the obese 67·7–71·6 (mean 69·1).

Our data indicates the wide variability in gross composition of adipose tissue in the human subject. The obese tend to have rather less water and more lipid with a slightly lower iodine number than the lean.

We are grateful to Professor Alan Kekwick for encouragement and to the Surgical and Pathological Staff for specimens.

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The Metabolism of L-Erythrulose by Rat-Liver Slices. By R. D. BATT,* F. DICKENS and D. H. WILLIAMSON. (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1*)

L-Erythrulose reacts, as a donor of active glycolaldehyde, with spinach transketolase (Horecker, Smyrniotis & Klenow, 1953) and its reduction with the diphosphopyridine nucleotide-linked xylitol dehydrogenase of guinea-pig liver has been reported (Hickman & Ashwell, 1959). The biosynthesis of L-erythrulose has been described in a system containing transketolase, carboxylase and hydroxypyruvate (Dickens & Williamson, 1956) and, recently, [1:2-$^{14}C_2$]erythrulose has been prepared by incubating [U-^{14}C]fructose 6-phosphate with glycolaldehyde and transketolase (Datta & Racker, 1959). The biosynthesis of erythrulose 1-phosphate from dihydroxyacetone phosphate and formaldehyde with phosphoketotetrose aldolase has been demonstrated in rat-liver preparations (Charalampous, 1954).

The metabolism of L-erythrulose by rat-liver slices has now been studied using the tetrose labelled with ^{14}C in the 1 or 4 positions. [1-^{14}C]-Erythrulose was prepared by the incubation of a purified yeast transketolase with glycolaldehyde and [3-^{14}C]hydroxypyruvate, obtained by the action of D-amino acid oxidase on DL-[3-^{14}C]serine (Negelein & Bromel, 1939). [4-^{14}C]Erythrulose was prepared by the action of transketolase on hydroxypyruvate and [2-^{14}C]glycolaldehyde, the latter substance being formed by the action of ninhydrin on the L-[3-^{14}C]serine remaining from the preparation of [3-^{14}C]hydroxypyruvate described above. The tetritols corresponding to [1-^{14}C]- and [4-^{14}C]erythrulose were prepared by reduction with hydrogen and platinum oxide.

The incubation of rat-liver slices with [1-^{14}C]-tetritol, [1-^{14}C]- and [4-^{14}C]erythrulose and the subsequent isolation and degradation of glucose using *Leuconostoc mesenteroides* followed the procedures described by Dickens & Williamson (1959). With [1-^{14}C]erythrulose, 13·8% of the ^{14}C appeared as carbon dioxide and 4·8% was incorporated into the glucose (44% in C-1; 42% in C-6). With [4-^{14}C]-erythrulose 4·9% of the ^{14}C was released as carbon dioxide and 1·4% was incorporated into the glucose (20% in C-1; 28% in C-4; 33% in C-6).

[1-^{14}C]Tetritol was metabolized to a lesser degree than [1-^{14}C]erythrulose; only 1·0% of the ^{14}C was released as carbon dioxide and 0·6% was incorporated into glucose.

When L-erythrulose was incubated with liver slices at least 80% of the tetrose was utilized and there was an accumulation (about 20%) of the corresponding tetritol in the incubation system.

Liver-enzyme preparations showing high fructokinase and glycerokinase activities were inactive with L-erythrulose.

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The Occurrence of Amino Acid-activating Enzymes in Rat-Liver Mitochondria. By VALDA M. CRADDOCK* and M. V. SIMPSON.* (*Department of Biochemistry, Yale University, New Haven, Connecticut, U.S.A.*)

The occurrence of protein synthesis in mitochondria has been established by the demonstration that liver mitochondria can carry out the incorporation of amino acids into total protein and into cytochrome *c*, and that calf-heart mitochondria can effect the net synthesis of this haem protein (Bates & Simpson, 1959). Studies on amino acid incorporation into microsomes have indicated an absolute requirement for the soluble fraction of the cell, a requirement which has been explained partly by its content of amino acid-activating enzymes. In view of the fact that the mitochondrial system is active without the addition of such a fraction (although liver mitochondria is stimulated by boiled soluble fraction), it was of interest to discover whether these enzymes are present within the mitochondria themselves. Experiments were carried out to test this possibility.

Two reactions were studied: the formation of amino acid hydroxamates, and an amino acid-dependent pyrophosphate–adenosine triphosphate (ATP) exchange. Six-times washed liver mito-

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chondria were disrupted by sonic vibration for 5 min. The sonicate was adjusted to pH 5, the precipitate was rehomogenized in tris (2-amino-2-hydroxymethylpropane-1:3-diol) buffer, pH 7·4, and the suspension recentrifuged. The final supernatant fluid, on incubation with ATP, $MgCl_2$, tris buffer, pyrophosphatase, hydroxylamine, with and without the addition of an L-amino acid mixture, showed amino acid-dependent hydroxamate formation. Other mitochondrial fractions showed hydroxamate formation which was not amino acid dependent.

In studying the exchange reaction, the mitochondrial preparation showing amino acid-dependent hydroxamate formation was incubated with labelled pyrophosphate, ATP, $MgCl_2$, tris buffer, KF, 2:4-dinitrophenol, in the presence and absence of an added L-amino acid mixture. The reaction was stopped by addition of trichloroacetic acid, and the ATP was isolated and counted. Addition of a complete amino acid mixture approximately doubled the percentage exchange/mg. of protein, the exchange varying linearly with the amount of protein added. Tryptophan and leucine were found to be mainly responsible for this enchancement of the exchange reaction.

An amino acid-dependent pyrophosphate–ATP exchange has been shown to occur in acetone powder preparations of rat-liver mitochondria (Reis, Coote & Work, 1959*a*, *b*) but was not found in mammary-gland mitochondria (Jones & Gutfreund, 1959).

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Bacterial Ribonucleoprotein Synthesized in the Presence of Chloramphenicol. By S. Dagley and J. Sykes. (*Department of Biochemistry, University of Leeds, Leeds* 2)

In the presence of chloramphenicol, bacterial synthesis of protein may be inhibited whilst that of ribonucleic acid (RNA) continues. The RNA thus accumulated has the same base composition as normal bacterial RNA (Horowitz, Lombard & Chargaff, 1958) but has sometimes been considered abnormal because it is rapidly degraded by the cells when freed from antibiotic (Neidhardt & Gros, 1957; Hahn, Schaechter, Ceglowski, Hopps & Ciak, 1957). We have shown recently that when cells of *Escherichia coli* are incubated with chloramphenicol and extracts from them are examined in the ultracentrifuge, peaks of large area at 18s and 14s appear in schlieren diagrams (Dagley & Sykes, 1959). Previous workers had not observed them (see Pardee, Paigen & Prestidge, 1957), probably because the accumulated RNA is present as a constituent of macromolecules sensitive to sonic vibrations. Thus they are rapidly broken down when vibrations pass through extracts made from chloramphenicol-treated cells disrupted in the Hughes press; and when whole cells are subjected to sonic vibrations for the minimum time required for any significant cell breakage, the extract obtained contains little or no material sedimenting at 14–18 s. The RNA accumulating by chloramphenicol action has been termed 'fast-moving' but in the light of present observations it would seem desirable to re-examine its electrophoretic properties; certainly its particle size is much greater, possibly by a factor of 50, than the 'acceptor RNA' from mammalian preparations (Zamecnik, Stephenson & Hecht, 1958). Since cell rupture by the Hughes press does not break down this 14–18 s material it may be isolated by differential centrifugation and when so prepared analyses gave RNA, 49%; protein, 51%; i.e. more RNA and less protein than in bacterial microsomal particles previously examined. Apparently osmotic shock also provides undegraded material (Aronson & Spiegelman, 1958).

The peaks which occur during chloramphenicol action sediment at about the same speed as small peaks detectable in extracts from untreated cells. Is the material 'normal' ribonucleoprotein (RNP) which accumulates, due to a blockage in its utilization? The following facts are presented against this view. (1) Normal (40 s) RNP is broken down in citrate solution; 'chloramphenicol RNP' is not. (2) Magnesium ions catalyse breakdown of the latter but stabilize or aggregate the former. (3) Release of the drug from whole cells results in an abolition, which is almost total, of peaks at 14–18 s and a considerable augmentation of the normal 29 s peak. It is the increase of this form of RNP which is to be correlated with recovery and growth (Dagley & Sykes, 1959).

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Localization of Some Glycosidases in the Mammalian Cell. By J. Conchie and G. A. Levvy. (*Rowett Research Institute, Bucksburn, Aberdeen, Scotland*)

It has been shown (Kerr & Levvy, 1951; Walker & Levvy, 1951; Walker, 1952) that mouse-liver β-glucuronidase is confined almost entirely to the mitochondria and microsomes, from which it can be released in a variety of ways, e.g. exposure to a hypotonic medium or treatment with the non-ionic surface-active agent, Triton X-100. The enzymes, α-mannosidase, β-galactosidase and β-*N*-acetylglucosaminidase (Conchie, Findlay & Levvy, 1959) have now been found to display a similar intracellular distribution in mouse liver, and the localization of all four glycosidases has been extended to mouse kidney and the mouse S 37 tumour.

Owing to variability in the performance of homogenizers, difficulties arise in standardizing suspensions of subcellular particles in isotonic media, and this problem becomes more acute when attempts are made to compare different organs. Neglect of this factor can nullify refined analyses of enzyme distribution by differential centrifuging. It was found best to compare preparations after analysis of the time factor in homogenizing, subcellular particles being separated by the method of Hogeboom (1955). Compared with liver, kidney cells were more resistant to homogenizing, but gave rise to more fragile cytoplasmic granules, whilst the tumour was tougher in both respects. Granules of different sizes appeared to disintegrate at different rates in the homogenizer.

When the homogenizing factor was eliminated in comparing results, all four enzymes displayed a similar pattern of distribution in kidney, one that closely resembled liver. In the tumour, β-glucuronidase and β-galactosidase were equally distributed between the cytoplasmic granules and the extraparticulate cytoplasm, whilst β-*N*-acetylglucosaminidase was confined to the subcellular particles, from which it was only released with difficulty. α-Mannosidase appeared to resemble β-*N*-acetylglucosaminidase in this respect, but with the former enzyme the results are complicated by instability towards prolonged homogenizing. It also displays anomalous behaviour with Triton X-100.

de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) have interpreted results for a number of other mammalian hydrolytic enzymes in terms of the 'lysosome', a specialized cytoplasmic granule of definite size, in which the enzymes are enclosed together. Explanations in terms of a multiplicity of less specialized particles are, however, equally valid, and more consistent with the present work.

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Some Early Biochemical Changes Accompanying Inhibition by Streptomycin of the Growth of *Staphylococcus aureus*. By R. Hancock (introduced by E. F. Gale). (*Department of Bacteriology, University of Manchester, Manchester* 13)

As a preliminary to investigating metabolic changes which could be responsible for the inhibition of growth of *Staphylococcus aureus* (strain Duncan) by streptomycin, it has been found that the bactericidal effect of this antibiotic is most rapid on cells growing exponentially under aerobic conditions in medium of pH 7·2; under these conditions over 90% of the cells lose colony-forming ability after exposure for 75 min. to 10 μg. of streptomycin sulphate/ml. If during this period of contact with streptomycin growth is prevented by withdrawal of essential amino acids or by lowering of the tem-

perature, or if the culture is made anaerobic, the rate of loss of viability is considerably reduced.

During the period in which growth inhibition by streptomycin becomes complete, no specific or differential inhibition has been observed of the rates of incorporation of ^{14}C-labelled precursors (amino acids or glucose) into any of the major cell fractions (pool, ethanol-soluble 'protein', protein, ribonucleic acid, deoxyribonucleic acid, cell-wall mucopeptide, and teichoic acid). Before growth inhibition becomes detectable, the rate of oxygen consumption of streptomycin-treated cultures decreases significantly. Manometric experiments using cells harvested from such cultures show that the value of Q_{O_2} (glucose) is about 70% of that of normal cells when growth inhibition is first detectable, and may, in fully inhibited cells, fall to 20% of normal.

Attempts have been made to search for changes in the pathways of glucose metabolism in streptomycin-treated cells by investigating chromatographically the distribution of radioactivity from [^{14}C]glucose among hot-water-extractable compounds and in metabolic end-products. Considerable differences between normal and streptomycin-treated cells have been found in the distribution of ^{14}C between intermediates of the glycolytic pathway and other products, and are being investigated in more detail. Similar differences have been observed in the pattern of incorporation of inorganic ^{32}P. These changes in glucose metabolism have not been observed in non-growing cells exposed to streptomycin.

DEMONSTRATION

Immuno-Diffusion on Cellulose Acetate. By R. Consden and J. Kohn. [*Rheumatism Research Unit (Medical Research Council), Taplow, Maidenhead, Berks*]

Cellulose acetate in membrane form is a satisfactory medium for immuno-diffusion purposes and has a number of advantages over agar (Consden & Kohn, 1959). The procedure for carrying out the Ouchterlony type of double diffusion on membranes is demonstrated. The technique consists in applying the antigen and the antiserum to the surface of a buffer or saline-impregnated cellulose acetate membrane. This is then placed under oil for a suitable period of time for the diffusion and precipitation process to take place. The oil is then removed, the excess protein washed out, and the cellulose acetate membrane stained for the demonstration of the precipitation pattern. Under favourable conditions the precipitation lines (revealed by staining) are very sharp and by rendering the stained membrane transparent with oil, greatly enlarged negatives of these lines may be easily prepared. Micro-immuno electrophoresis of the antigenic components is carried out as described by Kohn (1958), followed by application of the antiserum to the strip parallel to the electrophoretic direction. Diffusion is then allowed to proceed as in the Ouchterlony method. Components separated by preliminary electrophoresis may be transferred to another membrane and an Ouchterlony diffusion then carried out.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **389th Meeting** *of the Society was held in the Department of Chemical Pathology, King's College Hospital Medical School, London, on Saturday,* **19** *December, when the following papers were read:*

COMMUNICATIONS

The Xanthine Oxidase of Human Milk and Colostrum. By P. L. Bradley and M. Gunther. (*Wellcome Laboratories of Tropical Medicine, The Wellcome Building, Euston Road, London, N.W.* 1, *and Infant Welfare and Children's Department, University College Hospital, Gower Street, London, W.C.* 1)

It has been stated that human milk contains little or no xanthine oxidase (Modi, Owen & Proudfoot, 1959), and is to this extent unlike the milk of ruminants which is so rich in this enzyme. The failure to obtain precise measurements of xanthine oxidase activity in human milk is most likely due to the use of methods of insufficient sensitivity, for when we used a very sensitive procedure (Bauer & Bradley, 1956) we were able to determine the activity of this enzyme in all of about thirty samples of human milk and colostrum.

Briefly, the technique used depended upon anaerobic reduction of 2:3:5-triphenyltetrazolium chloride at pH 9·4 with hypoxanthine as substrate. The red triphenylformazan was extracted into acetic acid and toluene, and the optical density read at 495 mμ. As the reduction of tetrazolium proceeded in a linear manner in relation to time and amount of enzyme present, a unit of activity was referred to as that spectrophotometer reading which would be obtained if 1 ml. of sample (i.e. milk, colostrum, etc.) were allowed to react for 1 hr. In actual practice very much smaller samples were used.

By relating the xanthine oxidase activity to the time after parturition at which the sample was taken, a very unusual distribution was found. There was very little of the enzyme in pre-natal and immediate post-natal colostrum (0·1–1·0 unit), but the activity rose to a sharp peak at about the third day *post partum* (highest value, 8·8 units). Thereafter the activity fell just as rapidly until it reached a low plateau of about 1 unit of activity around the sixth or seventh day. This phenomenon was confirmed by taking serial samples covering the critical period from three mothers. In order to put these figures into relation with known activities, six samples of fresh cow's milk were assayed, and gave a mean activity of 21 units, with a range of 13 to 28.

Centrifugal fractionation of early secretion was also carried out. Large samples were subjected to three phases of centrifugation. (*a*) At 500 ***g*** for 20 min. there were two samples, the cream, and the deposit which consisted of cells and colostrum bodies. (*b*) At 6500 ***g*** for 20 min. the deposit consisted of cell fragments. (*c*) At 105 000 ***g*** for 30 min. there was an almost clear supernatant and a small gelatinous pellet, presumably composed largely of microsomes. Although some activity was seen in all fractions, by far the greatest amount of enzyme was found in the cream and the final supernatant. The 'microsomal' fraction had a comparatively high activity, but by volume it constituted only a very small portion of the total.

We are very much indebted to Dr A. L. Greenbaum of University College London, for help with the centrifugal fractionations, and to Dr T. R. Ashton of the Express Dairies, Ltd., who made available samples of fresh cow's milk.

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The Isolation of Bradykinin: A Plasma Kinin from Ox Blood. By D. F. Elliott, G. P. Lewis and E. W. Horton. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Bradykinin belongs to a group of peptides which stimulate smooth muscle and have vasodilator activity, and which have been given the generic name of plasma kinins (see Lewis, 1958). It is formed by the action of trypsin on plasma globulins (Andrade & Rocha e Silva, 1956; Rocha e Silva, Beraldo & Rosenfeld, 1949).

In the present investigation ox-serum proteins were fractionated by addition of ammonium sulphate, the solid precipitating between 33 and 45% of saturation being collected and salts removed by dialysis. After treatment at 37° with 0·1 N-hydrochloric acid, to inactivate bradykinin-destroying enzymes, the solution was incubated

with pure trypsin for 6 hr. The reaction was terminated by addition of boiling ethanol and the substances soluble in 75% ethanol were subjected to a short counter-current distribution to remove lipoid material and salt.

Further purification was carried out by elution chromatography on two successive carboxymethylcellulose columns in ammonium acetate buffer at pH 6·5 and 5. The solid remaining after evaporation of the buffer contained 10–25% of pure bradykinin.

Complete purification by paper chromatography or paper electrophoresis was hampered by the presence of magnesium in this peptide mixture; flame spectrographic analysis revealed the presence of approximately 1% by weight. It was therefore necessary to remove at least the greater part of the magnesium by performing the second carboxymethylcellulose-column fractionation in presence of ethylenediaminetetra-acetic acid. These results suggest that bradykinin has a high affinity for magnesium ions and this may have some bearing upon its mode of action.

The pure peptide was obtained by preparative paper electrophoresis. On acid hydrolysis bradykinin yielded serine, glycine, proline, phenylalanine and arginine in the molar proportions 1:1:2:2:2. The purity was confirmed by the presence of a single *N*-terminal residue, that of arginine. The biological activity of the pure polypeptide has been found to be comparable with that of histamine and acetylcholine.

The authors wish to express their thanks to Miss M. Kinsella and to Messrs R. Blakemore, A. W. Hemmings and E. Stevens for experimental assistance.

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Pathways of Glucose Oxidation in Retina. By MARGARET KERLY and M. ATAUR RAHMAN. (*Department of Biochemistry, University College, London, W.C.* 1)

A number of workers (Futterman & Kinoshita, 1959; Cohen & Noell, 1959; Holt, Lüth, Hallman, Holt & Voelker, 1959) have recently studied the pathways of glucose oxidation in retina during incubation with [1-^{14}C]glucose and with [6-^{14}C]glucose. Their findings suggest that, although glucose can be metabolized by the hexose monophosphate shunt, this pathway does not play a part in oxidation in aerobic conditions. Since in retina, addition of bicarbonate increases both oxidation and glycolysis, it was of interest to determine the specific activity of both carbon dioxide and lactic acid formed during incubation of retina in media containing labelled glucose buffered with phosphate or with bicarbonate. Portions of ox retina (400–500 mg. wet wt.) were incubated for 45 min. at 38° in 2 ml. of Krebs–Ringer buffered either by phosphate or by bicarbonate and containing 20 μmoles of [^{14}C]glucose (750 counts/min./μmole).

The following values were found for [1-^{14}C]-glucose and [6-^{14}C]glucose, respectively (counts/min./μmole). (*a*) Phosphate buffer: carbon dioxide, 55 and 56; lactic acid, 320 and 455. (*b*) Bicarbonate buffer: carbon dioxide, 18 and 14; lactic acid, 402 and 534. (The amounts of carbon dioxide and lactic acid formed were, in the phosphate medium 6 and 11 μmoles, and in the bicarbonate medium 17 and 18 μmoles, respectively.)

Although the values for the specific activity of the carbon dioxide give no evidence of oxidation by the shunt pathway, the values for lactic acid suggest that in both media more is formed from C-4, C-5 and C-6 of glucose than from C-1, C-2 and C-3. The ratio of isotope in lactic acid from [1-^{14}C]-glucose and [6-^{14}C]glucose was the same after incubation in both media. It will be observed that in all experiments the specific activity of the carbon dioxide was lower than that to be expected had it been produced only by direct oxidation of the added glucose. It is likely that ^{14}C-labelled carbon dioxide was diluted by carbon dioxide produced by oxidation of endogenous substrate and by further oxidation of recyclized glucose fragments. Fixation of carbon dioxide, which is known to be high in retina (Crane & Ball, 1951), is probably also a complicating factor; the high specific activity of the lactic acid in the bicarbonate experiments indicates that some fixation of ^{14}C-labelled carbon dioxide occurred.

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The Reactions of Reduced Flavine Nucleotides with Oxygen. By H. GUTFREUND. (*National Institute for Research in Dairying, Shinfield, near Reading*)

One of the most interesting problems about the reactions of flavoproteins is the remarkable change in reactivities of the flavine nucleotides as they are bound to different oxidizing and electron-transport enzymes. In our recent study of xanthine oxidase (Gutfreund & Sturtevant, 1959) we have shown that dithionite reduces this enzyme very much more slowly than it reduces free flavine adenine dinucleotide (FAD). We have also shown that the enzyme binds oxygen prior to the re-oxidation of the xanthine-reduced flavine group. It seemed important to know more about the reactivities of free FAD and flavine mononucleotide (FMN) before continuing a comparison of those flavoproteins which react with oxygen, with those of the respiratory chain, which do not react directly with oxygen.

The reactions of FAD and FMN reduced with dithionite were studied at 25° in the stopped-flow fast-reaction spectrophotometer at 450 mμ. Excess dithionite was removed by slow addition of buffer saturated with oxygen, prior to mixing the flavine solutions with buffer (0·1 M-citrate–phosphate, pH 6·1) saturated with oxygen at various pressures. It was found that the reaction constants (k) were first-order with respect to flavine concentration but were not proportional to oxygen concentration. Linear plots were obtained when $1/k$ was plotted against $1/[O_2]$, giving half-optimal oxygen concentrations of 0·135 mM and 0·27 mM for FAD and FMN, respectively. The maximum velocity (k_2) was 29·0 sec.$^{-1}$ for FAD and 20·8 sec.$^{-1}$ for FMN. When both the reduction of FMN and its re-oxidation were carried out in D_2O, k_2 was reduced to 10·8 sec.$^{-1}$ and the half-optimal concentration of oxygen was reduced to 0·11 mM. In contrast to the findings of Beinert (1956) no optical density change could be detected at 565 mμ during the course of any of these reactions.

We conclude from these observations that oxygen is bound to the reduced flavine perhaps across the reactive hydrogens, prior to the oxidation step, which involves the removal of the two hydrogen atoms. Only the final oxidation step, characterized by k_2, is likely to be slowed down when the hydrogens are replaced by deuterium atoms. This gives further support to the suggestion of Gutfreund & Sturtevant (1959) that deuterium should be a useful indicator to distinguish between hydrogen- and electron-transport steps.

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Effects of Salicylate and 2:4-Dinitrophenol on the Metabolism of [^{14}C]Glucose and [2-^{14}C]Acetate in Isolated Rat Tissues. By M. J. H. SMITH and V. MOSES. (*Department of Chemical Pathology, King's College Hospital Medical School, London, S.E.* 5)

Chopped preparations of isolated rat tissues (kidney, liver, brain, testis and heart muscle) were incubated with either [^{14}C]glucose or [2-^{14}C]acetate; the ethanol–water-soluble intermediates were then separated by paper chromatography. The radioactive spots were detected by means of X-ray film and counted with an end-window counter. The ^{14}C from both substrates was found to be incorporated into maltose, sugar phosphates, nucleotides, amino acids and organic acids. The general pattern with each substrate was similar for all the tissues, although there was some variation in the percentage distributions of radioactivity in the various fractions.

When the experiments were repeated in presence of either 5 mM-salicylate or 0·5 mM-2:4-dinitrophenol there was a decreased incorporation of total radioactivity from both labelled substrates into the soluble intermediates of all the tissues, except when brain was incubated with the radioactive glucose. The percentage incorporations of the ^{14}C from both the [^{14}C]glucose and the [2-^{14}C]acetate into the sugar phosphates, maltose and nucleotide fractions, and from the [2-^{14}C]acetate only, into the amino acids, were reduced. Increased percentages of radioactivity from both labelled substrates occurred in the organic acids.

The relation of these findings to other known effects of salicylate and dinitrophenol on metabolism were discussed.

We are grateful to the Empire Rheumatism Council and to the Board of Governors of King's College Hospital for grants towards the cost of the work.

[^{14}C]Glucose Metabolism in Baker's Yeast and the Effects of 2:4-Dinitrophenol and Sodium Salicylate. By V. MOSES and M. J. H. SMITH. (*Department of Chemical Pathology, King's College Hospital Medical School, London, S.E. 5*)

Baker's yeast, obtained commercially, was grown for 3 hr. at 25° in a glucose–ammonia–salts medium. The cells were resuspended in 2 mM-phosphate buffer, pH 5·0. [^{14}C]Glucose was added and the cell suspension shaken at 25° for 3 hr. Samples were removed at intervals from 5 sec. to 3 hr., and the cells killed and extracted with ethanol. The extracts were fractionated by paper chromatography and radioactive substances located by radioautography and counted with an end-window Geiger–Müller tube.

Similar experiments were performed in presence of 8 mM-salicylate and 0·1 mM-2:4-dinitrophenol, which were the concentrations found to produce maximum stimulation of endogenous oxygen consumption and minimum oxidative assimilation of added glucose in the yeast.

After 3 min. incubation both salicylate and dinitrophenol caused a decreased incorporation of total ^{14}C into the soluble intermediates and, more specifically, reduced the activity in the sugar phosphates, uridine diphosphate glucose, nucleotides, free sugars (mainly trehalose), and organic acids (dinitrophenol only), while more ^{14}C was found in the amino acids. The 3 min. sample represents a short incubation period, much of the ^{14}C still being present in the [^{14}C]glucose supplied as the substrate and in early intermediates such as the sugar phosphates and uridine diphosphate glucose. At a later period (3 hr.), when almost all the added glucose had disappeared from the medium, the presence of 2:4-dinitrophenol and salicylate had resulted in a decrease in the total ^{14}C present in the cell extracts, a moderate decrease in the sugar phosphates, and little change in uridine diphosphate glucose, nucleotides, and organic acids. However, while most of the ^{14}C in the control sample appeared in trehalose, with dinitrophenol and salicylate present the amounts in trehalose were relatively small, but large amounts of ^{14}C were found in the amino acids.

Most of these differences in the distribution of ^{14}C can be interpreted on the basis of dinitrophenol and salicylate being primarily uncouplers of oxidative phosphorylation. Hence, an interruption of a source of energy for synthesis results in an inhibition of trehalose formation and an accumulation of amino acids owing to a blockage of protein synthesis.

We are grateful to the Empire Rheumatism Council and the Board of Governors of the King's College Hospital for grants towards the cost of the work.

An Insulin Antagonist Associated with Human Albumin. By J. VALLANCE-OWEN and M. DELORIS LILLEY. (*Department of Medicine, King's College, University of Durham, Newcastle upon Tyne* 1)

Insulin antagonism has been found to reside in the albumin fraction of plasma from uncontrolled insulin-requiring diabetics and from normal subjects (Vallance-Owen, Dennes & Campbell, 1958*a*). This antagonism appears to depend on the presence of the pituitary gland, as plasma albumin prepared from hyperphysectomized subjects is devoid of insulin antagonism (Vallance-Owen, Dennes & Campbell, 1958*b*).

Albumin has now been prepared in quantity from normal freeze-dried plasma by the trichloroacetic acid–ethanol procedure of Debro, Tarver & Korner (1957) and, as previously, the rat-diaphragm technique (Vallance-Owen & Hurlock, 1954) used for detecting the antagonistic activity.

The principle responsible for insulin antagonism associated with normal albumin has now been separated from the protein. The active protein has been treated in several ways. (*a*) The albumin in 0·8–1 % aqueous solution has been heat-coagulated by boiling for 5 min., preferably at pH 5·5. (*b*) The dry protein has been left in contact with an organic liquid, either methanol, acetone or butanol for at least 24 hr. at room temperature. (*c*) An 0·8 % albumin solution in N-acetic acid or N-ammonia has been allowed to stand for 3 days at room temperature.

Prolonged dialysis, up to 7 days, of these protein suspensions or solutions against distilled water permitted the passage of the antagonist into the diffusate. The latter was satisfactorily evaporated *in vacuo* without loss of antagonistic activity.

The active diffusate from heat-coagulated albumin shows no loss of activity on digestion with the proteolytic enzymes, pepsin and trypsin.

Ethanol extraction of albumin under reflux results in complete loss of antagonistic activity. Also extraction of the protein with an *iso*octane–acetic acid solvent mixture does not lead to concentration of the antagonist in the fatty acid residue.

The insulin antagonist therefore appears to withstand heating at 100° at pH 5·5 for 5 min., and at room temperature is stable over the pH range 2·6–11·6. It is not adversely affected by organic liquids at room temperature and is unlikely to be a protein or fatty acid but, as it is readily diffusible, it is presumably a compound of low molecular weight.

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Pathway of Trehalose Biosynthesis in the Locust Fat Body. By D. J. Candy and B. A. Kilby. (*Department of Biochemistry, University of Leeds*)

Trehalose is the predominating carbohydrate in insect haemolymph and the locust fat body has been identified as a site of trehalose biosynthesis (Candy & Kilby, 1959). The pathway of biosynthesis has now been largely elucidated by the use of ^{14}C-labelled D-glucose as a substrate and the examination of deproteinized incubation mixtures by paper chromatography and radioautography. A dialysed supernatant fraction of adult *Schistocerca* fat body was obtained which would convert glucose into trehalose in the presence of adenosine triphosphate (ATP) and uridine diphosphate glucose (UDPG). A number of other products were also obtained and identified by co-chromatography, some of these being intermediates, as shown by their early rise in concentration and subsequent fall during the incubation period. Amongst these intermediates were glucose 6-phosphate and trehalose phosphate, the trehalose phosphate peak appearing later in time than the glucose 6-phosphate peak. Another intermediate was fructose 6-phosphate and other final products were α-glycerophosphate and glycerol. Presumably these latter compounds were produced by a pathway other than that for trehalose biosynthesis.

Glucose 6-phosphate served as a substance for trehalose biosynthesis if the enzyme preparation was fortified with UDPG, addition of ATP being unnecessary. Addition of ATP, however, decreased the yield of trehalose by diverting more substrate into a side path leading to α-glycerophosphate.

The enzyme preparation hydrolysed trehalose phosphate to trehalose and inorganic phosphate, the rate of hydrolysis being greater than for any other sugar phosphate tested, suggesting the presence of a specific phosphatase. The enzyme had a pH optimum at 6·25, showed maximum activity with mM-Mg^{2+}, was completely inhibited by mM-ethylenediaminetetra-acetic acid but was unaffected by 10 mM-fluoride.

Uridine triphosphate activated the system in place of UDPG, although not to the same extent. Uridine triphosphate and glucose 1-phosphate in presence of a partially purified preparation of fat body were found to form a substance chromatographically identical to UDPG.

The pathway of trehalose biosynthesis from glucose in the locust appears to occur by initial phosphorylation of the glucose followed by subsequent transfer of a glucosyl unit to glucose 6-phosphate from UDPG by an enzyme similar to that previously described in yeast (Leloir & Cabib, 1953). The product, trehalose phosphate, is then hydrolysed to trehalose.

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The Oxidation of Hydroxylamine in Cell-free Extracts of *Nitrosomonas europaea*. By D. J. D. Nicholas* and O. T. G. Jones*. (*Long Ashton Research Station, University of Bristol*)

Cell-free preparations of *Nitrosomonas* oxidize hydroxylamine to nitrite in presence of a suitable acceptor such as phenazine methosulphate, pyocyanine, methylene blue, benzyl viologen or mammalian cytochrome *c*, but ferricyanide, 2:3:6-trichloroindophenol dye, coenzymes I and II, methyl viologen, or glutathione are without effect (Nicholas & Jones, 1959). When cytochrome *c* was used as the acceptor there was a phosphate requirement but at higher concentrations phosphate inhibited the oxidation of hydroxylamine, as did a number of other anions, including pyrophosphate, citrate, selenate, tungstate and arsenate. The phosphate inhibition, which occurred when cytochrome *c* was the acceptor, is competitive, but there was no effect

* Members of the Scientific Staff of the Agricultural Research Council seconded to the Long Ashton Research Station.

with phenazine. Hydrazine, known to inhibit nitrite production from hydroxylamine in whole cells (Lees & Hoffman, 1952,) had a similar effect on the cell-free system when cytochrome *c* was used, but it was without effect, however, when phenazine methosulphate was the acceptor. Although these extracts did not oxidize ammonia, its addition stimulated the conversion of hydroxylamine into nitrite. A 50-fold purification of the enzyme system has been achieved by ammonium sulphate precipitation and treatment on columns of diethylaminoethylcellulose.

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An Androgen-Activated Pyridine Nucleotide Transhydrogenase in Prostatic Tissue. By D. N. Baron, Marion B. R. Gore and D. C. Williams. (*Department of Chemical Pathology, The Royal Free Hospital and School of Medicine, London, W.C.* 1)

The endocrine treatment of malignant disease poses the problem of how steroid hormones influence cell growth and metabolism. Villee (1955) postulated the activation by oestradiol-17β of a soluble diphosphopyridine nucleotide (DPN)-dependent *iso*-citric dehydrogenase from human placenta. Talalay & Williams-Ashman (1958) showed that the actual hormone-sensitive enzyme system was a pyridine nucleotide transhydrogenase catalysing hydrogen transfer from reduced triphosphopyridine nucleotide to DPN. They postulated that this transhydrogenase activity was due to hydroxy steroid dehydrogenases, but Hagerman & Villee (1959) have separated these systems. Oestrogen-activated transhydrogenases have been demonstrated in human breast, breast cancer tissue, and placenta, and androgen-activated transhydrogenase in rat liver.

The present work demonstrates an androgen-activated transhydrogenase in rat prostate and in histologically normal human prostate (separated from adenomata removed surgically) but not in prostatic adenomata. Cell-free fractions were obtained by ultracentrifugation of fresh homogenized tissue and were tested for transhydrogenase activity, with and without added hormone, using the acetylpyridine analogue of DPN (APDPN) (Hurlock & Talalay, 1958). APDPNH can be measured spectrophotometrically in the presence of DPNH.

$$\text{DPNH} + \text{APDPN} \xrightarrow{\text{transhydrogenase}} \text{APDPNH} + \text{DPN}$$

The activity could be increased, usually about two-fold, by the addition of catalytic quantities of androsterone, but not by oestrogens. The androgen-activated transhydrogenase of liver and the oestrogen-activated transhydrogenase of placenta were similarly demonstrated.

The observed basal transhydrogenase activity which was present before the addition of steroid hormone was found to increase on standing at room temperature with a corresponding decrease in the degree of activation with added steroid. The same result could be produced more rapidly by incubation with β-glucuronidase. The increase in basal transhydrogenase with time could be largely inhibited by the addition of the β-glucuronidase inhibitor, saccharo-1:4-lactone (Levvy, 1952). These results suggest that endogenous β-glucuronidase liberates steroid from its glucuronide on standing and that this steroid is partially responsible for the basal transhydrogenase activity. Thus the apparent differences between basal and activated transhydrogenase levels may, in part, be due to preparative conditions allowing hydrolysis of the steroid glucuronide.

The prostate extracts were also tested for hydroxy steroid dehydrogenase activity using stoicheiometric quantities of steroid hormone (Hurlock & Talalay, 1958) but none was detected.

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The Fatty Acids of Human Faecal Lipids. By A. T. JAMES, JOAN P. W. WEBB and T. D. KELLOCK. (*National Institute for Medical Research, Mill Hill, London, N.W. 7, and Central Middlesex Hospital, Park Royal, London, N.W. 10*)

It is usually accepted that the main factor controlling the fatty acid composition of human faecal lipids is the composition of the dietary fat. Quantitative analysis of the methyl esters of the bound and free fatty acids from human faeces has shown the presence of unusual fatty acids (frequently in high concentration) not existing in the diet. Large amounts of these acids are excreted when the faecal fat level is high and can rise to 10–15 g./day in cases of steatorrhea.

The structures of these unusual acids have been determined by studying their chromatographic behaviour on two types of column (James, 1959) and also by conventional chemical techniques. The first unusual component is a mixture of C_{18} monoenoic acids having the double bond in positions 4:5, 5:6, 7:8, 10:11, 11:12 and 12:13.

The chromatographic behaviour of the second unusual component could not be correlated with that of any mono-, di-, tri-, tetra-, penta- or hexa-unconjugated unsaturated acid. This was confirmed by determination of its ultraviolet absorption spectrum. Its chromatographic behaviour could only be explained on the basis of the existence of a very polar group in the molecule, the most likely one being a hydroxyl group. The chromatographic effects of a hydroxyl group were determined by comparison of the behaviour of oleic and ricinoleic acids (12-hydroxy-Δ^9-octadecenoic acid), and suggested that the original compound was a saturated C_{18} hydroxy acid. Oxidative degradation and characterization of the resultant mono- and di-carboxylic acids with the gas chromatogram (James & Webb, 1957), showed that the hydroxyl group was at C-10.

Whilst alteration of the type of dietary fat gives rise to change of composition of the faecal fat in no case are they identical.

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Amidase Production by *Pseudomonas aeruginosa*. By M. KELLY and PATRICIA H. CLARKE. (*Biochemistry Department, University College, London, W.C. 1*)

A strain of *Pseudomonas aeruginosa* able to grow on acetamide as sole source of carbon and nitrogen was obtained by serial subcultivation of strain NCTC 8602 on a medium containing inorganic salts, glucose and acetamide. Washed suspensions of the new strain, 8602/A, hydrolysed acetamide rapidly if acetamide had been added to the growth medium, but if acetamide were omitted the amidase activity was slight. The acetamide-grown cells hydrolysed propionamide at a higher rate than acetamide and had slight activity towards formamide and butyramide.

The amidase could also be induced by the addition to the growth medium of *N*-methylacetamide which was not a substrate of the enzyme. Amidase activity at the end of the growth period was maximal with 10 mM-*N*-methylacetamide and little or no induction could be detected at mM concentrations. Cyanacetamide, thioacetamide, fumaramide and malonamide were neither substrates nor inducers of the enzyme.

N-Methylacetamide was added as an inducer to log-phase cultures of strain 8602/A growing on an ammonium salt medium with succinate as carbon source. A short initial lag was followed by a linear increase in enzyme activity/mg. dry wt. of cells which continued at a steady rate for at least 3 hr., at an inducer concentration of 5 mM. At lower inducer concentrations the rate of increase fell off after periods of time depending on the initial inducer level. When the inducer concentration was between 1·5 and 2·5 mM this was followed by a second period of rapid increase in enzyme. Below an inducer concentration of 1·5 mM this second phase of enzyme synthesis did not occur, and there was a steady fall in the enzyme concentration per cell as the enzyme originally synthesized was diluted out by the growth of new cells. A possible explanation for these results has been devised.

One of us (M. K.) is indebted to the Agricultural Research Council for a Postgraduate Studentship.

Biosynthesis of 2-Methoxyoestriol by Rat-liver Preparations. By R. J. B. KING. (*Department of Biochemistry, University of Edinburgh*)

In agreement with similar experiments with oestrone and oestradiol-17β (Ryan & Engel, 1953), it has been found that the metabolism of oestriol by rat-liver slices is markedly stimulated by molecular oxygen. When oestriol is incubated with rat-liver slices in Krebs–Ringer phosphate and oxygen for 2 hr. two phenolic metabolites (I and II) are obtained. Both are non-ketonic.

Compound I, formed in about 10% yield, is less polar than oestriol, migrating on paper between 16-*epi*oestriol and 17-*epi*oestriol in chloroform–formamide. It has been identified as 2-methoxyoestriol by comparison of its infrared and ultraviolet spectra, m.p. and optical rotation with those of authentic 2-methoxyoestriol.

Compound II, formed in about 18% yield, is more polar than oestriol, having R_F 0·05 in acetic acid–water–ethylene dichloride (oestriol, R_F 0·65). Compound II is unusual in that it gives a blue colour with the Folin–Ciocalteu reagent in the absence of alkali (Davies & Mitchell, 1954).

Using rat-liver homogenates it was observed that for oestriol metabolism and formation of I and II, diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) were needed. TPN is better than DPN but the best results are obtained with DPN plus TPN. Formation of I will not proceed in the absence of methionine and Mg^{2+} and is stimulated by adenosine triphosphate (ATP). The amount of oestriol metabolized is only slightly affected by these three factors but when they are omitted formation of II is increased threefold.

Incubation of II with homogenates supplemented with methionine, ATP and Mg^{2+} results in the formation of I, but it is not formed in the absence of methionine.

These observations and the infrared and ultraviolet spectra and the behaviour with the Folin–Ciocalteu reagent are compatible with the view that II is 2-hydroxyoestriol.

It is suggested that 2-methoxyoestriol, which has also been isolated from urine as a metabolite of [16-^{14}C]oestradiol-17β (Fishman & Gallagher, 1958), is formed in the liver as follows:

$$\text{Oestriol} \xrightarrow[O_2]{\text{DPN or TPN}} \text{2-Hydroxyoestriol} \xrightarrow[(O_2)]{\text{Methionine, ATP, } Mg^{2+}} \text{2-Methoxyoestriol}$$

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The Association of ^{14}C-Labelled Vitamin D_2 with Serum Proteins. By KAREEN J. INNES CHALK and E. KODICEK. (*Dunn Nutritional Laboratory, University of Cambridge, and Medical Research Council*)

Vitamin D, after incubation with human serum, moves electrophoretically in the albumin and α-globulin region, suggesting that it is attached to the proteins during its transport (Thomas *et al.* 1959).

Using ^{14}C-labelled vitamin D_2, more detailed information was obtained on the distribution of radioactive material in relation to the protein fractions. Uniformly ^{14}C-labelled vitamin D_2 (10 μg., specific activity 0·396 μc/mg.) was added in 20 μl. of propylene glycol to 4 ml. of rat serum and incubated for 30 min. at 37°. After electrophoresis of the sample on a starch-supporting medium (Kunkel & Slater, 1952), 1 cm. segments were analysed for protein with the Folin–Ciocalteu reagent (Peters & van Slyke, 1932), for ^{14}C and for antirachitic activity. The segments were extracted successively with saline, with light petroleum and, after boiling with methanol, with ethanol–diethyl ether (3:1, v/v). Portions of the saline extracts were taken for protein analysis.

The extraction from starch segments of ^{14}C-labelled material, combined or not with serum, was tested with these three solvents. In absence of serum, light petroleum extracted all the ^{14}C, but in its presence only 50% was removed. To obtain complete extraction of the material, when associated with serum, boiling with methanol followed by treatment with ethanol–diethyl ether was required. Thus it would appear that in presence of serum proteins the solubility in lipid solvents was reduced. Saline extraction was not a good indication of water solubility of the radioactive material, since, even in the absence of serum, about 20% was extractable from starch.

On electrophoresis a correlation was found between the peaks of radioactive material and those of albumin and α-globulin. The albumin fraction was associated with 34% and the α-globulin with

39% of the radioactive material, but the concentration of ^{14}C was four times higher in the α-globulin than in the albumin fraction because of their respective amounts of serum. This suggests that α-globulin may be a more efficient carrier of vitamin D than albumin. The remaining radioactive material did not move from the point of application, indicating that it was not associated with electrophoretically mobile components.

Reversed-phase paper chromatography on paraffin-impregnated paper (Kodicek & Ashby, 1954), and biological assay (Bourdillon, Bruce, Fischmann & Webster, 1931) of the fractions obtained after electrophoresis showed that only about 30% of the radioactive material was biologically and chemically identifiable as vitamin D.

We wish to thank Dr E. M. Cruickshank for help in the biological assays. One of us (K. J. I. C.) is in receipt of an M.R.C. scholarship.

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Separation of a Peptide with Insulin-like Properties from Preparations of Ox Growth Hormone.

By A. K. Huggins* and J. H. Ottaway.* (*Department of Biochemistry, University College, London, W.C.* 1)

The presence of insulin-like activity in growth-hormone preparations has been reported (Park, Brown, Cornblath, Daughaday & Krahl, 1952; Ottaway & Bulbrook, 1955), and partial separation of the activity from growth-hormone preparations, using ultrafiltration, was reported by Ottaway & Paul (1957). Separation and complete purification of a peptide with insulin-like properties has now been accomplished by electrophoretic techniques.

Ox growth hormone prepared by Wilhelmi's (1955) method was subjected to electrophoresis (3–4 v/cm.) for 48 hr. at 1–3° in a 60 cm. starch block, equilibrated with 0·05M-ethylamine acetate at pH 10·0. The apparatus was modified from that described by Fønss-Bech & Li (1954). After passage of the current, 1 cm. sections of the block were extracted twice with buffer at pH 10. Measurements of the optical density of the combined extracts at 275 mμ revealed the separation of a number of small peaks, chiefly on the anodic side of the main protein peak, which had remained stationary. The extracts corresponding to these peaks were freeze-dried to remove the volatile buffer, and tested for insulin-like activity using the isolated rat diaphragm incubated in Krebs–Ringer bicarbonate with added acetate (Ottaway, 1960). Only the slowest moving anodic peak was found to possess activity.

The material was further analysed by paper electrophoresis at pH 8·5, and was found to contain two ninhydrin-reacting components originating from the growth hormone. One, with an isoelectric point at pH > 10 was inactive; the other, with an isoelectric point at about pH 6·5, had insulin-like activity. It was separated in a pure state by paper electrophoresis at pH 8·5 in ammonium acetate buffer, followed by freeze-drying to remove salt. The residue was shown to contain N, and on hydrolysis gave a mixture of amino acids, from which basic and aromatic amino acids are apparently completely absent. The absorption spectrum shows maxima at 220 and 275 mμ.

The peptide gives significant insulin-like activity in the rat-diaphragm assay at a concentration of 0·004 μg./ml., and the injection of 10 μg. into starved rabbits produces a slight but long-lasting hypoglycaemia. It has no detectable adrenocorticotrophic hormone activity.

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The Distribution of Radioactive Iron between Ferritin and Haemosiderin in Rat Tissues.
By J. Veronica Fulton and W. N. M. Ramsay. (*Department of Biochemistry, University of Edinburgh, Edinburgh* 8)

Two well-characterized iron-storage substances are known: ferritin, which is soluble, not heat-coagulable below 80°, and precipitated by half-saturation with ammonium sulphate (Granick, 1946) and haemosiderin, which is insoluble even in dilute salt solutions. The proportions of the two in liver and spleen have been studied by Kaldor (1958) in rats and rabbits and by Shoden, Gabrio & Finch (1953) in rabbits. The latter workers felt that ferritin and haemosiderin were functionally indistinguishable, but some of their results suggest that ferritin takes up radioactive iron more rapidly than does haemosiderin.

Five weeks after the subcutaneous administration of 100 mg. of iron as iron–dextran complex, mature male rats were given injections of tracer amounts of ^{59}Fe-labelled ferric citrate. The concentration and radioactivity of ferritin and total non-haem iron (TNHI) were determined at intervals thereafter. The differences were assumed to be due to haemosiderin in the TNHI fraction. The analytical procedure involved the extraction with hot perchloric acid of precipitates formed by the addition of ammonium sulphate (to 55% saturation) to crude aqueous suspensions of finely chopped, frozen tissues (TNHI) and to the non-heat coagulable fraction of such suspensions (ferritin). The iron in the extracts was co-ordinated with $\alpha\alpha'$-dipyridyl and precipitated with Na_2HgI_4 (Feigl, 1949). The precipitate was washed, dissolved in acetone, transferred to copper planchettes and counted at infinite thinness with an end-window counter. The removal of blood from the tissues by perfusion was unnecessary because ^{59}Fe present as haemoglobin caused only 2·3% interference.

It was found that in all three tissues, but especially in liver, the radioactive iron at first appeared mainly in the ferritin. Later there was some degree of redistribution. Under the conditions of these experiments, ferritin undoubtedly takes up iron more rapidly than does haemosiderin.

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The Inhibition of Antibody Formation *in vitro*.
By R. W. Dutton.* (*School of Medicine and Dentistry, University of Rochester, N.Y., U.S.A.*)

Rabbit-spleen cells removed 2 or 3 days after an anamestic antigenic stimulation *in vivo* will incorporate ^{14}C-labelled amino acid into antibody when incubated *in vitro*. Cells isolated 3 days after stimulation synthesize antibody at a constant rate for 48 hr. (Vaughan, Dutton, Dutton, George & Marston, 1960). In contrast, cells isolated at 2 days synthesize antibody at a rate which increases during the first 24 hr. incubation. This synthesis can be partially inhibited by 5-bromouracil deoxyriboside (BUDR) and the inhibition can be reversed by thymidine (Dutton, Dutton & Vaughan, 1959).

These observations have been extended. In the experiments now reported spleen cells isolated 2 days after stimulation have been used and the antibody synthesized in 48 hr. measured.

Low concentrations of BUDR markedly reduced the amount of antibody synthesized (50% inhibition at 6 μg./ml.). Much higher concentrations, however, were only slightly more inhibitory (75% inhibition at 800 μg./ml.). The reversal of inhibition by thymidine appeared to be competitive over the ranges studied.

5-Fluorouracil deoxyriboside (FUDR) also inhibited antibody formation, although it was less effective than BUDR. The degree of inhibition showed the same lack of sensitivity to the concentration of the inhibitor and the inhibition was reversed by thymidine.

When BUDR or FUDR were added 24 hr. after the start of the incubation period, at a time when the rate of antibody formation had already increased, there was little or no inhibition of antibody synthesis during the following 24 hr. period.

It thus appeared, from these experiments with the '2-day' spleens, that the inhibitors were more effective in preventing the increase in capacity to synthesize antibody than antibody synthesis *per se*. The development of new antibody-forming capacity was dependent on some metabolic function of thymidine. On this interpretation, it might have been expected that the inhibitors would have little effect on the synthesis of antibody by

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cells isolated at 3 days, since here the rate of antibody synthesis does not increase on incubation. In fact this is only partially the case. Although the inhibitors were considerably less effective the same relationship between concentration and inhibition was found, in that low concentration of inhibitor produced an inhibition which was not greatly exceeded at much higher concentrations. Further work is needed to explain these findings.

This work was supported, in part, by a U.S. Public Health Service Grant.

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The Lipolytic Activity of Adipose Tissue. By D. S. Robinson. (*Sir William Dunn School of Pathology, University of Oxford*)

When intact rat-epididymal fat bodies are incubated *in vitro*, unesterified fatty acids are released into the incubation medium. Although several of the factors controlling the extent of this release are known (Gordon & Cherkes, 1958; Reshef, Shafrir & Shapiro, 1958; Knobil, 1959) the possible role of the clearing factor lipase has not been investigated hitherto.

In the present study it has been found that, when fat bodies from starved rats are incubated *in vitro* in albumin solution, the production of unesterified fatty acids is little reduced in the presence of protamine sulphate, sodium pyrophosphate or M-sodium chloride solutions. All these inhibit the action of the clearing factor lipase. Production is as rapid as pH 6·1 as at pH 8·1 and is not increased when heparin or chyle is added to the incubation medium. Thus there is no evidence that the release of unesterified fatty acids from the fat bodies under these conditions involves the clearing factor, or indeed any other, lipase.

When fat bodies from rats which have been fed their normal diet and given intravenous injections of glucose solution in addition, are incubated *in vitro* in albumin solution, the release of unesterified fatty acids is low when chyle is absent from the medium. In the presence of chyle, however, at pH 8·1 the release of unesterified fatty acids is much greater (four- to five-fold) than that occurring with fat bodies from starved rats and is raised further when heparin is present. In presence of heparin, lipase is released from the fat body into the incubation medium. At pH 6·1, production of unesterified fatty acids is very low and is also low at pH 8·1 in presence of the inhibitors mentioned above. The release of unesterified fatty acids from chyle triglyceride under these conditions, therefore, appears to be due to the action of clearing factor lipase present in epididymal fat tissue and the findings suggest that the enzyme may be concerned in the transport of triglyceride to the fat depots in the carbohydrate-fed animal. The results agree with those of Hollenberg (1959) and Cherkes & Gordon (1959).

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Lipid Synthesis by Human Leucocytes *in vitro*. By A. A. Buchanan (introduced by T. A. J. Prankerd). (*Medical Unit, St Mary's Hospital, London, W.* 2)

Lipid synthesis by erythrocytes *in vitro* has been recently reported by several investigators (Altman, Watman & Salomon, 1951; Altman, 1953; James, Lovelock & Webb, 1957; Marks, Johnson, Hirschberg & Banks, 1958; Rowe, 1959; Turner, 1958). In these experiments blood, sometimes with the 'buffy coat' removed, has been incubated *in vitro* after addition of sodium [*carboxy*-^{14}C]acetate to the plasma. After incubation the cells and plasma have been extracted for lipid and the radioactivity of the extract estimated. The lipids have been fractionated and most fractions shown to contain ^{14}C.

In the present experiments leucocytes have been separated by dextran sedimentation (Davis, Wilson & Spun, 1958) and by differential red-cell haemolysis with saponin (Schwartz, Grazia, Cheney, Hasenfus & Cohen, 1956). Erythrocytes have been obtained free from leucocytes by filtration of the blood through cotton wool (Fleming, 1926) and multiple centrifugation with removal of the top layers of cells. The resultant cells were incubated at 37° in plasma previously obtained from a portion of the blood labelled with sodium [*carboxy*-^{14}C]-acetate. After incubation the cells and plasma were

extracted for lipid by the method of Folch, Lees & Sloane-Stanley (1957); the lipid was weighed and its radioactivity estimated.

It was shown that in all cases leucocytes were 100–1000 times as active as erythrocytes in lipid synthesis. In some experiments where erythrocytes were obtained uncontaminated with leucocytes the erythrocytes were found to be completely inactive.

The rate of labelling of the lipid fractions in both cells and the plasma in which the cells were incubated is initially extremely rapid, falling after 4–6 hr. incubation, after which very little further labelled lipid is formed. Pure red cells incubated in sodium [*carboxy*-^{14}C]acetate-labelled plasma appeared to be unable to utilize the acetate.

The lipid synthesis was shown to be temperature dependent, being completely inhibited at 4°. Plasma incubated with sodium [*carboxy*-^{14}C]acetate showed no labelling of the lipid fraction.

Glycolytic and respiratory inhibitors (sodium fluoride, sodium azide, 2:4-dinitrophenol, potassium cyanide) in concentrations sufficient completely to inhibit aerobic respiration did not completely inhibit lipid synthesis by leucocytes.

In order to show whether or not erythrocytes have any ability to synthesize lipid, 1 ml. samples of blood were taken. From half the samples the erythrocytes were haemolysed with saponin in cold 0·6% sodium chloride (leaving the total white-blood cells from these samples intact). The total cells (red- and white-blood cells) from the control samples were washed in the same concentration of saponin in cold 0·9% sodium chloride (this does not haemolyse the erythrocytes). Both samples were incubated with similar volumes of sodium [*carboxy*-^{14}C]acetate-labelled plasma. After incubation it was found that the leucocytes alone synthesized lipid in exactly the same amounts as did the total cells (red- and white-blood cells), showing that the erythrocytes do not synthesize any of the labelled lipid found after incubation of whole blood.

When sodium [*Me*-^{14}C]acetate was added to the plasma before incubation, in the concentration normally used for [*carboxy*-^{14}C]acetate, exactly similar quantities of labelled lipid were formed.

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Lipid Biosynthesis in Human Blood: the Incorporation of Acetate into Lipids by Different Types of Human-Blood Cell. By C. E. Rowe, A. C. Allison and J. E. Lovelock. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Human-blood cells can incorporate *in vitro* acetate (James, Lovelock & Webb, 1959; Lovelock, James & Rowe, 1960) and inorganic phosphate (Rowe, 1959) into their lipids. Acetate is also incorporated into the leucocytes of human patients with leukaemia (O'Donnell, Ottolenghi, Malbin, Denstedt & Heard, 1958).

Human blood in acid–citrate–glucose was incubated with sodium [*Me*-^{14}C]acetate or sodium [*carboxy*-^{14}C]acetate at 35° for periods up to 5 hr. The cells were then fractionated by differential centrifugation in 28% (w/v) bovine albumin in saline. The uppermost fractions contained the highest proportions of leucocytes and the lowermost mature erythrocytes with the least contamination by leucocytes or reticulocytes. The lipids were extracted, fractionated and saponified.

The incorporation of radioactivity into the unsaponifiable lipid was proportional to the number of leucocytes in the fraction. Moreover, a high incorporation of acetate into the fatty acids of neutral saponifiable lipid was associated with a high leucocyte count. The incorporation into the fatty acids of the phospholipids showed some scatter.

In other experiments fractionation of cells, followed by resuspension in plasma, preceded incubation with sodium [*Me*-^{14}C]acetate. Incorporation of acetate into unsaponifiable lipid again depended on the number of leucocytes.

The simultaneous increase with time of the specific radioactivities of the lipid components of leucocytes and red cells in normal blood were measured over the course of 5 hr. The cells were fractionated by passage of a suspension in saline

containing 0·1 N-calcium chloride through Ballotini beads, thereby reducing the number of leucocytes to red cells by a factor of 12.

The results combine to show that leucocytes are responsible for most, if not all, of the synthesis of unsaponifiable lipid. The neutral saponifiable fat had the highest specific activity of the lipid fractions. It would appear that there is some synthesis of this by the red cells.

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DEMONSTRATIONS

Partition Chromatography of Steroid Esters. By R. W. H. Edwards. (*Department of Chemical Pathology, King's College Hospital Medical School, London, S.E.* 5)

Techniques for the partition chromatography of steroid derivatives of 'low polarity' were demonstrated. Steroids included acetoxy- and formoxy-derivatives of mono- and di-hydroxylated androstane and pregnane, with and without centres of unsaturation and ketonic substituents. The solvent systems used are based on two-phase formic acid–light petroleum mixtures.

A Simple Scanning Device for Radioactivity on Paper Chromatograms. By N. J. Holness and J. F. Fowler. (*Department of Chemical Pathology, King's College Hospital Medical School, London, S.E.* 5)

The apparatus was designed for the location and semiquantitative measurement of radioactivity on one-dimensional paper chromatograms. The counting chamber is of the gas-flow, windowless type, scanning both sides of a paper-chromatogram strip. The counting chamber is mounted on a modified recording clinical ratemeter (Type D 4130, Labgear Ltd.) and the chromatogram strip is drawn through the counting chamber by the chart-drive mechanism of the ratemeter. At optimum sensitivity the ratemeter can detect a spot containing 0·3 mμc of ^{14}C on the paper.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **390th Meeting** *of the Society was held in the Department of Biochemistry, London Hospital Medical College, on Saturday,* 23 *January* 1960, *when the following papers were read:*

COMMUNICATIONS

The Proteinase Activity of Gastric Mucosal Extracts from Patients with Carcinoma of the Stomach. By W. H. Taylor.* (*Department of Clinical Biochemistry, Radcliffe Infirmary, University of Oxford*)

Extracts of gastric carcinomatous tissue from eight patients have been compared with extracts of the uninvaded gastric mucosa obtained from the same gastrectomy specimens. Curves relating pH to activity for the digestion of plasma protein by extracts of uninvaded mucosa exhibited, in four patients, two maxima, at pH 2·1–2·3 and at pH 3·3–3·7. These are similar to the maxima shown by gastric mucosal extracts of normal subjects (Taylor, 1956, 1959*a*). In three patients, three maxima occurred, at pH 1·5–2·1, 2·6–2·8 and 3·5–3·8, resembling the maxima shown by a minority of normal subjects and by a majority of patients with peptic ulcer (Taylor, 1959*b*). The remaining patient gave a curve with two maxima, at pH 1·6 and 3·1, which resembled curves obtained from extracts of normal pyloric mucosa (Taylor, 1959*c*).

The carcinoma extracts of seven patients, in contrast, digested plasma protein with only one maximum, at pH 3·2–3·4, and the degree of digestion at pH 2 was slight, ranging from 0 to 40% of that at the maximum, whereas with uninvaded mucosa the activity at each of the maxima is roughly the same. The eighth patient differed from the others in that the carcinoma had invaded, but not ulcerated, the mucosal surface, and the curve of pH versus activity did not differ significantly from that of the uninvaded mucosa.

Differences were also observed for the digestion of egg albumin. Whereas extracts of uninvaded mucosa resembled normal mucosal extracts in exhibiting one maximum at pH 1·5–1·8 and sometimes a second maximum at pH 2·5, the carcinoma extracts failed to digest this substrate between pH 1·5 and 5·0.

In two patients, extracts of secondary carcinomatous deposits from omental lymph glands gave curves relating pH to proteolytic activity resembling those of the primary growth.

The proteolytic activity of the carcinoma extracts does not seem to arise from smooth-muscle cells incorporated into the growth nor from reactive fibrous tissue, but to be a property of the adenocarcinomatous cells themselves, in which they differ from the normal cells from which they are derived.

This work was in part supported by a personal grant from the Medical Research Council.

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Further Observations on a Fat-Mobilizing Substance Extractable from Human Urine. By G. L. S. Pawan. (*Medical Unit, Middlesex Hospital, London, W.* 1)

The urine of normal fasting human subjects and of certain patients was shown to contain a fat-mobilizing substance which could be extracted by weak alkali after precipitation with ethanol (Chalmers, Kekwick, Pawan & Smith, 1958). The method of extraction has since been improved and further observations made on the chemical nature and biological properties of this material.

An ethanol–benzoic acid precipitate of the urine sample was washed with ethanol and subsequently extracted in weak alkali. The crude alkaline extract was treated with ethanol and the precipitate after washing repeatedly with ethanol was taken up in alkali. After centrifugation in the cold, the alkaline extract was neutralized, adsorbed on to oxidized cellulose powder, and the active substance subsequently eluted with alkali.

The active material contained about 8% nitrogen, and after acid hydrolysis the following amino acids were detected: histidine, phenyl-

alanine, leucine, serine, cystine, aspartic acid and a trace of alanine. The active material was thermostable up to 80° in 0·1 N-sodium hydroxide but was destroyed by boiling for 2 min. After peptic digestion some activity remained, but activity was completely destroyed by trypsin and by chymotrypsin.

In the mouse, this substance increases fat mobilization and catabolism and produces a loss in body weight and body fat. It also causes a reduction in blood sugar. The pituitary gland appears to be necessary for its production. It is not growth hormone nor adrenocorticotrophic hormone.

This work was carried out in collaboration with Professor Alan Kekwick and Dr T. M. Chalmers, to whom I would like to express thanks for constant advice and encouragement.

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Lipid Synthesis in Fowl Blood. By JOAN P. W. WEBB, A. C. ALLISON and A. T. JAMES. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

The *in vitro* synthesis of lipids by human whole blood has been described previously (James, Lovelock & Webb, 1959), and further studies have shown the relative contribution of the various cellular components to this synthesis (Rowe, Allison & Lovelock, 1960).

A similar series of experiments has now been carried out using fowl blood to study the synthesis of lipids by nucleated red cells. Fowl blood was incubated *in vitro* with [*Me*-^{14}C]acetate under the same conditions as those used with human blood and the resulting radioactivity of a number of lipid fractions was investigated.

It was found that acetate was incorporated with the long-chain saturated and unsaturated fatty acids of the phospholipids and neutral fat fractions of both cells and plasma. The most active of the lipid fractions was the triglycerides, followed by the phospholipids and then the cholesterol esters. Fowl blood differs from human blood in respect of (i) its ability to synthesize a number of non-saponifiable sterol-like substances from acetate, and (ii) its ability to incorporate fatty acids into cholesterol esters.

Further experiments were carried out using fractions of fowl blood enriched in mature erythrocytes, young erythrocytes and leucocytes. The lipids from the leucocyte-rich fraction were found to be the most highly labelled, but there was considerable activity in both erythrocyte fractions, the young erythrocytes being more active than the mature. The fatty acids from these fractions were separated on the gas chromatogram and the pattern of labelling of the individual acids was found to be similar in all three groups of cells.

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Observations on the Mechanism of the Aerobic Control of Glycolysis in Brain Mitochondrial Fraction. By R. BALAZS and D. RICHTER. (*Medical Research Council Neuropsychiatric Research Unit, Whitchurch Hospital, Cardiff*)

The mechanism of the aerobic control of glycolysis previously described in preparations of brain mitochondria (Balazs, 1959) has now been further investigated.

Aerobic inhibition (25–35 %) of lactate formation in a system containing the standard medium (6 mM-magnesium sulphate, 10 mM-potassium dihydrogen phosphate, 20 mM-2-amino-2-hydroxymethylpropane-1:3-diol, 2 mM-ethylenediaminetetra-acetate, 25 mM-nicotinamide at pH 7·2) with 10 mM-hexose diphosphate, 0·33 mM-diphosphopyridine nucleotide, 2 mM-adenylic acid and mM-adenosine triphosphate ('hexose diphosphate-fortified system') was not removed by 1·4 μM-antimycine-A or 0·2 mM-dinitrophenol. The site of the control was previously shown to be at the triose phosphate dehydrogenase step (Balazs, 1959); this was confirmed by showing that addition of the crystalline enzyme abolished the inhibition. Cysteine and glutathione were without effect.

In a system containing the standard medium with 6 mM-pyruvate and 10 mM-glucose ('unfortified system'), both glucose consumption and lactate formation were inhibited aerobically by about 50 and 80 %, respectively. The inhibition was abolished by 2 mM-cyanide, mM-azide and 16 μM-

2-heptyl-4-hydroxyquinoline *N*-oxide. Blocking the respiratory chain with 3 mM-amytal or 1·4 μM-antimycine-A also removed the inhibition, which was not restored by feeding in electrons above the block with succinate or ascorbate and cytochrome *c*. The aerobic control of glycolysis was also abolished by uncouplers of phosphorylation (0·1 mM-dinitrophenol and 0·02 mM-dicoumarol). Addition of crystalline triose phosphate dehydrogenase had no effect.

Thus two mechanisms of control apparently operate under different conditions. (*a*) One mechanism, insensitive to antimycine-A and dinitrophenol, is a specific aerobic inhibition of triose phosphate dehydrogenase; (*b*) the other, unaffected by adding triose phosphate dehydrogenase but sensitive to respiratory inhibitors and phosphorylation uncouplers, may be connected with the processes of oxidative phosphorylation. Both types of inhibition are completely reversible by changing the gas phase.

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The Mechanism of the Action of Androgenic Hormones on Mouse-Liver Catalase. By D. H. Adams. (*Cancer Research Department, London Hospital Medical College, London, E.* 1)

In mouse liver, catalase is distributed between large granules and extra-particulate cytoplasm (EPC). The level of activity in the granules is approximately the same in both sexes, while the activity in the EPC is higher in males than in females (Adams & Burgess, 1957). Possibly therefore two separate catalase systems are present in liver cells, one reacting to hormones and the other not. However, the evidence from spectroscopic and fractionation studies, and studies of catalase–hydrogen peroxide complex II formation, in a variety of species (Bonnichsen, 1947; Chance, 1950; Deutsch & Seabra, 1955; Adams & Burgess, 1959*a*), suggests that only a single catalase is present in liver. This provides no indication why only the EPC catalase is affected by hormones.

Recently Adams & Burgess (1959*b*) showed that during the incubation of liver slices, catalase migrated from the large granules into the EPC. Catalase migration into the surrounding medium took place when granules were incubated in 0·25 M-sucrose solution. Since no evidence of damage to or rupture of the granule membranes was found, it was concluded that the intact membranes were permeable to catalase. It seemed possible therefore that liver contains a single catalase which is produced in the large granules and is then distributed between the granules and EPC by migration through the granule membranes. The EPC level will then depend broadly on the granule level, the permeability of the granule membranes, and the rate of destruction in the EPC. Taking the simplest possible view, it may be considered that the relative levels of catalase in granules and EPC (granule:EPC ratio) will provide an approximate index of granule permeability. The injection of cysteine, thiourea or 3′-methyl-4-dimethylaminoazobenzene into mice resulted in transient falls in catalase level, without change in the granule:EPC distribution ratio. This was true in both males and females despite their differing initial ratios (about 5·0 in females and 3·3 in males). The injection of 17-methyltestosterone into females also gave a transient fall in catalase activity, but after 48 hr. the distribution ratio changed to the male value. It appears therefore that androgens act on catalase by bringing about an intracellular redistribution of the enzyme, and this is consistent with the theory that this is brought about by increasing the permeability of the granule membrane.

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A Blood-group-specific Sialomucopolysaccharide possessing Virus-receptor Activity. By A. Pusztai and W. T. J. Morgan. (*Lister Institute of Preventive Medicine, London, S.W.* 1)

Water-soluble blood-group-specific substances are neutral mucopolysaccharides built up from four sugars (L-fucose, D-galactose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine) and eleven or twelve amino acids. Purified preparations, which are fully active serologically, can contain as little as 0·3% sialic acid. Mucopolysaccharides which contain the above-mentioned sugars and a major component of sialic acid, although believed to occur in human secretions and tissue digests,

have not been firmly identified (Odin, 1959). Gibbons (1959) has, however, recently described 'blood-group-like' substances of animal origin which contain about 14% of sialic acid.

A blood-group-specific Le^a-substance obtained from a human-ovarian-cyst fluid, after purification through 90% phenol, fractionation from saturated ammonium sulphate at 60° and precipitation from water between 48 and 55% ethanol, analysed as follows: $[\alpha]_D^{20°} - 24°$ in water; N, 4·8; fucose, 8·4; total hexosamine, 26·6; reducing sugars (as glucose, after complete hydrolysis with 0·5N-hydrochloric acid at 100° for 16 hr.), 51·3; sialic acid (as *N*-acetylneuraminic acid), 19%. The sedimentation constant, S_{20}, was 5·7 (*c*, 0·55% in buffer, pH 8) and was determined by Dr R. A. Kekwick. The substance migrated anodically at pH 8 as a homogeneous material, the mobility being 7·5 (cm.^{2} sec.$^{-1}$ v^{-1} × 10^{-5}). This is much greater than for blood-group substances free from sialic acid. The material belongs to a class of carbohydrate–amino acid complexes which can be provisionally called sialomucopolysaccharides. The substance possessed powerful group-specific Le^a character and was a powerful inhibitor of influenza virus haemagglutination (indicator strains, ROB type B and ASH type A).

The sialic acid component did not prevent decomposition of the material with crystalline papain or ficin, as earlier reported for similar group-specific mucopolysaccharides free from sialic acid (Pusztai & Morgan, 1958). The sialic acid was quantitatively released by (1) 0·1N-sulphuric acid at 80° for 1 hr., (2) incubation at pH 2 and 37° for 24 hr., and (3) purified receptor-destroying enzyme obtained from *Vibrio cholerae* filtrates. The sialic acid recovered was identical with *N*-acetylneuraminic acid.

After removing sialic acid the substance was electrophoretically homogeneous, migrated more slowly, and gave the following analytical values: $[\alpha]_D^{20°} - 24°$ in water; N, 5·2; fucose, 9·0; total hexosamine, 29·4; reducing sugars (as glucose, measured as above), 55%. Its relative viscosity was about 20% less than the original sialomucopolysaccharide. The removal of sialic acid did not influence the Le^a specific activity, but caused complete loss of capacity to inhibit viral haemagglutination.

The isolation of the Le^a-substance from the crude ovarian-cyst fluid by means of digestion with pepsin at pH 2, fractionation from saturated ammonium sulphate at 60°, and from dimethyl sulphoxide with ethanol gave a material which was devoid of sialic acid but otherwise closely similar in composition to materials described above or prepared earlier (see Morgan, 1959).

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Occurrence of Phosphorylcholine and Phosphorylethanolamine in Goat Placenta. By M. W. Neil. (*Department of Biochemistry, London Hospital Medical College, London, E.* 1)

The analysis of goat placenta for phosphoric acid and its esters was briefly reported by Neil, Walker & Warren (1957). These analyses showed that 50–70% of the acid-soluble phosphorus compounds obtained from goat placenta consisted of an acid-stable phosphoric acid monoester fraction which contained practically no esterified hexose, pentose, glycerol or glyceric acid. Preliminary qualitative analysis indicated the presence of esterified choline in the fraction. Since considerable amounts of phosphorylcholine and phosphorylethanolamine occur in rabbit uterus, and perhaps also in human, cow and pig uteri (Cawkwell, 1958), it seemed of interest to re-investigate this fraction.

Resolution of the fraction by paper chromatography (phenol–ammonia) followed by analyses of phosphate-containing areas of the paper for total phosphorus revealed that 68% of the phosphorus of the fraction was located in the region of R_F 0·85 and about 8% in the region of R_F 0·35. These R_F values correspond to phosphorylcholine and phosphorylethanolamine, respectively.

The fraction was hydrolysed for 30 min. in N-hydrochloric acid at 100° and for 24 hr. in 2N-hydrochloric acid at 123°, followed by analyses for free choline by the periodide method of Appleton, La Du, Levy, Steele & Brodie (1953). No choline was liberated by the short hydrolysis, but choline equivalent to 68% of the total phosphorus of the fraction was liberated by the long hydrolysis. The conclusions drawn from the results of paper chromatographic, phosphorus and choline analyses were that all the choline in the fraction was present as phosphorylcholine and that this constituted 34–48% of the acid-soluble phosphorus compounds extracted from goat placenta.

Analyses for ethanolamine and serine were carried out after exhaustive hydrolysis of the

fraction, using phosphomonoesterase, by the fluorodinitrobenzene method of Axelrod, Reichental & Brodie (1953). The results indicated the absence of serine and the presence of ethanolamine in the hydrolysate, equivalent to about 10% of the total phosphorus of the fraction. These results were confirmed by paper chromatographic analysis (butanol–water, Mellon, Korn & Hoover, 1953) of the dinitrophenyl derivatives formed by reaction of fluorodinitrobenzene with the phosphatase hydrolysate.

The functions of phosphorylcholine and phosphorylethanolamine present in relatively large amounts in goat placenta are obscure. It would be interesting to investigate the possibilities that the embryos of some species may not be able to synthesize these esters in sufficient quantity for adequate phospholipid synthesis, and that the placental esters may represent a store ensuring an adequate supply of choline for the embryo, since choline is known to be essential for growth.

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Identity of the Metabolites of Hexoestrol Accumulated by the Genital Organs of Sheep. By J. Burgos-Gonzalez and R. F. Glascock. (*National Institute for Research in Dairying, University of Reading*)

Tritium-labelled hexoestrol ($7{\cdot}5 \times 10^7$ counts/min./μg.) was injected into female sheep (1 μg./kg. body weight) who were killed 4 hr. later when the concentration of radioactivity in the genital organs was expected to be at a maximum (Glascock & Hoekstra, 1959). After the addition of 10 mg. of carrier hexoestrol the genital organs were reduced to a pulp and thoroughly extracted with ethanol–ether (3:1, v/v) at pH 7. A phenolic fraction was then prepared from the extract. In one experiment in which the radioactivity in the uterus corresponded to 0·03 μg. of hexoestrol, 89·4% of it was extracted at pH 7 and of this all but 8% appeared in the phenolic fraction. To half of it (in benzene) was added 738 mg. of carrier and the solvent removed to yield 925 mg. of residue (I) which must have contained 79·3% of hexoestrol. After dilution of 46·8 mg. of I to 87·8 mg. with more carrier the mixture (II) had a specific activity of 1020 counts/min./mg. Pure hexoestrol (m.p. 188°) recrystallized from it had a specific activity of 1160 counts/min./mg. (calc. from II: 1142 counts/min./mg.). Again when 100 mg. of I was diluted to 332 mg. with carrier the hexoestrol (III) recrystallized from it had a specific activity of 582 counts/min./mg. (calc. from II: 600 counts/min./mg.). The diacetate prepared from it and recrystallized had a specific activity of 415 counts/min./mg. (calc. from III: 435 counts/min./mg.). Thus all the radioactivity in the phenolic fraction extracted at pH 7 followed the chemistry of hexoestrol.

Qualitative confirmation was obtained by chromatography of the phenolic fraction on alumina-impregnated paper (Glascock & Pope, 1960) when only one radioactive spot of R_F 0·6 (the same as that of hexoestrol) was obtained. In another experiment in which 30% of the radioactivity remained unextracted by ethanol–ether the tissue was digested in N-sodium hydroxide. No radioactivity could be extracted from it with ether at pH 7, one-fifth was extracted at pH 4 and the rest at pH 1. It is known that hexoestrol glucuronide is extracted at pH 4 (Smith & Williams, 1948) and that sulphate is hydrolysed at pH 1, and again chromatography of combined hydrolysed extracts on paper produced only one spot of R_F 0·6. As these two derivatives, however, are soluble in water it remains to be explained why they were not extracted by the first ethanol–ether treatment.

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Some Observations on Phosphotriose Isomerase. By C. W. PARR and MARY WHITTAKER. (*Department of Biochemistry, London Hospital Medical College, London, E.* 1)

There are indications that the various phosphoaldose isomerases function by similar reaction mechanisms. Thus, experiments using deuterium and tritium as markers have established a similarity of action for phosphoglucose, phosphomannose and phosphotriose isomerases (Bloom & Topper, 1956; Topper, 1957; Rieder & Rose, 1959). Also, both phosphoribose and phosphoglucose isomerases are powerfully inhibited by the phosphoaldonic acids derived from their respective substrates (Axelrod & Jang, 1954; Parr, 1956). It seemed of interest to examine whether phosphotriose isomerase was also inhibited by the corresponding phosphoaldonic acid, i.e. 3-phosphoglyceric acid.

3-Phosphoglyceric acid, and a number of other likely competitive inhibitors, were tested for their effect on the triose phosphate isomerase reaction. Such inhibitions as were observed were small and in no way comparable with those reported for phosphoribose and phosphoglucose isomerases. A possible explanation is as follows. Glucose 6-phosphate and ribose 5-phosphate are present very largely as ring structures, although presumably they have to pass transiently through an acyclic form before becoming attached as complexes to the enzyme surface. Phosphogluconate and phosphoribonate, on the other hand, are present entirely in the acyclic form. Thus these inhibitors compete with relatively low concentrations of acyclic phosphoaldose substrate. The triose phosphates do not exist in cyclic forms and consequently this situation cannot arise.

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Protein Synthesis in Protoplasts of *Bacillus megaterium*: the Passage of ^{14}C-Labelled Amino Acids through Phospholipid Fractions. By G. D. HUNTER and R. A. GOODSALL. (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W.* 3)

Hunter, Brookes, Crathorn & Butler (1959) recently suggested that lipids might play a part in protein synthesis by *B. megaterium*. A study has now been made of the labelling of lipid fractions when ^{14}C-labelled amino acids are incubated under growth conditions with protoplasts of this organism. After the incubations were completed, the whole protoplasts were extracted with methanol, leading to simultaneous lysis of the protoplasts and solubilization of the bulk of the lipids. The neutral lipid fractions, which contained very little ^{14}C, were removed from the combined amino acid–phospholipid fraction by chromatography on silicic acid columns. The amino acids were separated from the phospholipid fraction by paper chromatography.

The phospholipid fractions were always relatively heavily labelled after short incubations, and acid hydrolysis following a mild alkaline treatment led to the recovery of the original ^{14}C-labelled amino acid added. When the weight of ^{14}C-labelled amino acid used in the original incubation was small, then the label in the phospholipid fraction fell rapidly with time. On the other hand, when the weight of added amino acid was large in relation to the weight of protoplasts used, up to 7% of the added label was accumulated in this fraction. When, however, the addition of the labelled amino acid was followed after a short time with a large amount of unlabelled amino acid, an almost quantitative transfer of label from the phospholipid fraction into protein took place.

The phospholipid fraction is being further fractionated by paper chromatography on paper impregnated with silicic acid. The label is associated with material having the properties of a phospholipid but with a larger R_F than the common phospholipids in the usual solvent systems.

The radioactive-labelling of the phospholipid fraction does not seem to represent a simple transport process, as chloramphenicol prevented radioactivity appearing in this fraction without hindering the accumulation of radioactive amino acids by the protoplasts. Phospholipid–amino acid complexes may be concerned in the transfer of amino acids from the site of amino acid activation to the final site of protein synthesis, possibly ribosome particles attached to the cytoplasmic membrane (McQuillen, Roberts & Britten, 1959).

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Synthesis of Allyl Pyrophosphates from Mevalonate with Liver Enzymes and their Relation to Biosynthesis of Squalene. By DEWITT S. GOODMAN* and G. POPJÁK. (*Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W.* 12)

The synthesis from [2-^{14}C]mevalonate of acid-labile hydrophilic derivatives of dimethylallyl alcohol, geraniol, farnesol and of nerolidol with soluble liver enzymes has been reported (Popják, 1959). These substances had properties similar to those described by Lynen, Eggerer, Henning & Kessel (1958) for farnesyl pyrophosphate synthesized from mevalonate with yeast enzymes. The substances formed with liver enzymes have now been identified as allyl pyrophosphates.

The allyl pyrophosphates were formed when a soluble protein fraction of rat-liver homogenates, precipitable with ammonium sulphate between 30 and 60% saturation, was incubated with DL-[2-^{14}C]-mevalonate, adenosine triphosphate and Mg^{2+}. The yield from DL-mevalonate was 25–30%; when the optically active 5-phospho[2-^{14}C]mevalonate, prepared with liver mevalonic kinase (Levy & Popják, 1959), was used as substrate the yield of the allyl pyrophosphates was 75%. The pyrophosphates were extracted from the enzymic incubations (pH 7·3) with collidine and their aqueous solution prepared as described by Popják (1959); they were purified from contaminating nucleotides by precipitation of these at 4° with 90% ethanol. The allyl pyrophosphates resist hydrolysis by aqueous N-potassium hydroxide at 70–80° for 1 hr. They are stable at room temperature in aqueous solution down to pH 5·5, below which they gradually cleave into inorganic pyrophosphate (identified by the use of purified yeast pyrophosphatase) and alcohols. At pH 1–2 the cleavage is rapid and complete. The percentage cleavage of the allyl pyrophosphates plotted against pH below pH 5·5 gave a sigmoid-shaped curve, suggesting that the undissociated allyl pyrophosphoric acid is the unstable species. The allyl pyrophosphates are slowly hydrolysed at pH 8·3 by snake venom (*Naja naja*) phosphatases.

[^{14}C]Allyl pyrophosphates were prepared from about 200 μmoles of optically active 5-phospho-[2-^{14}C]mevalonate; 88% of the radioactivity of the [^{14}C]allyl pyrophosphates was associated with farnesyl and nerolidyl pyrophosphate. It was found that these sesquiterpenoid derivatives were converted into squalene in 75–80% yield under anaerobic conditions in the presence of washed liver microsomes, Mg^{2+} ions and reduced triphosphopyridine nucleotide (TPNH); with reduced diphosphopyridine nucleotide the yield of squalene was smaller than with TPNH. Addition of soluble enzymes to the microsomes reduced the yield of squalene and caused some conversion of the allyl pyrophosphates into carboxylic acids. Spectrophotometric assays showed that 1 mole of TPNH was used per mole of squalene synthesized.

A hypothesis concerning the mechanism of the final stages of squalene biosynthesis, based on these findings, has been advanced by Cornforth & Popják (1959).

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Sequences of Nucleotides in Deoxyribonucleic Acids. By K. BURTON. (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford*)

Diphenylamine (2%, w/v) in 66% (v/v) formic acid at 30° degrades deoxyribonucleic acid (DNA) to adenine, guanine, inorganic phosphate and a complex mixture of pyrimidine nucleotides. These nucleotides are the pyrimidine nucleotide sequences that were originally attached at each end to a purine nucleotide (Burton, 1956; Burton & Petersen, 1957, 1960). When the terminal phosphomonoester groups have been removed by a suitable enzyme (prostatic phosphomonoesterase) about 16 of the pyrimidine nucleotides can readily be separated by two-dimensional chromatography on paper, thus providing the basis of a simple method for the study of the frequencies of the corresponding sequences of nucleotides (Burton & Petersen, 1960).

This procedure was first applied to calf-thymus DNA and has now been applied to DNA from four other sources, namely *Escherichia coli*, salmon sperm, herring testes and the sperm of a sea urchin, *Echinus esculentis*. Each DNA shows several differences from the others and from the frequencies calculated for a randomly arranged polynucleotide of the same base composition. The amount of deoxycytidine (representing the frequency of the base sequence purine–cytosine–purine) is lowest in calf-thymus DNA, where it is about 70% of that for a random arrangement. The amount of thymidine

is notably high in the products from herring DNA (118% of the random). The dipyrimidine material containing one deoxycytidine and one thymidine residue is separated by the chromatography into the two isomers, deoxycytidylyl (3′ → 5′)thymidine and thymidylyl (3′ → 5′)deoxycytidine. The relative amounts of these two isomers are 0·9, 1·7, 1·4, 1·6 and 0·8:1, respectively, in DNA from *Escherichia coli*, salmon, calf, herring and *Echinus esculentis*.

There is, in calf-thymus DNA, a slight tendency for nucleotides to be bunched into polypyrimidine and polypurine sequences, as is indicated both by the amount of inorganic phosphate released by diphenylamine (28% of the total phosphorus) and by the amount of pyrimidines liberated as mono- and di-pyrimidine fragments (40% of the total pyrimidines). The other specimens of DNA do not show this tendency; the corresponding values are close to 25 and 50%, which are expected for a random arrangement.

The results do not give any evidence for a bias against the tricytidylyl, trithymidylyl or tetrathymidylyl sequences. However, for all five samples of DNA, the amounts of the tri- and tetranucleotide fragments containing 2 or 3 moles of thymine per mole of cytosine are only about 70% of those expected for a random arrangement.

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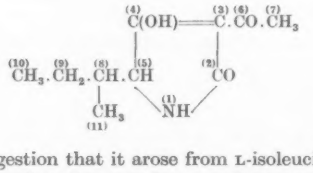
The Biosynthesis of Tenuazonic Acid. By C. E. Stickings and R. J. Townsend. (*Department of Biochemistry, London School of Hygiene and Tropical Medicine, University of London*)

Tenuazonic acid, a metabolite of the mould *Alternaria tenuis* (Rosett, Sankhala, Stickings, Taylor & Thomas, 1957) is 3-acetyl-5-*sec*.-butyltetramic acid (Stickings, 1959):



The suggestion that it arose from L-isoleucine and two molecules of acetate has now been confirmed.

Addition of sodium [*carboxy*-^{14}C]acetate to the normal Czapek–Dox culture medium led to the isolation of radioactive tenuazonic acid (3·9% incorporation of tracer). Acid hydrolysis of the tenuazonic acid yielded C-6 and C-7 as acetic acid, C-2 as carbon dioxide, and 3-amino-4-methylhexan-2-one (Stickings, 1959). Alkaline hypoiodite then gave C-3 as iodoform, and isoleucine. Treatment with ninhydrin gave C-4, obtained as carbon dioxide, and the resulting 2-methylbutyraldehyde was oxidized to the acid and degraded stepwise by the Schmidt and iodoform reactions to give each of the remaining carbon atoms separately. Of the total activity, 96% was present in nearly equal amounts in C-2 and C-6, the remaining 4% activity being shared by C-4 and C-10. All other C-atoms had no activity.

Strassman & Weinhouse (1955) have shown that L-isoleucine derived from protein synthesized by *Torulopsis utilis* is similarly labelled (in the carboxyl and terminal-methyl carbon atoms), when [*carboxy*-^{14}C]acetate is added to the medium. This is in agreement with the biosynthesis of isoleucine from acetaldehyde (or pyruvate) and α-oxobutyrate. Biosynthesis of the isoleucine-derived part of tenuazonic acid therefore appears to follow the same pathway. The activity at C-4 and C-10 is far lower than at C-2 and C-6 because the former do not arise exclusively from acetate.

Growth curves showed that tenuazonic acid was produced rapidly in surface culture on Czapek–Dox medium during the first two weeks of growth. The level then fell, but rose again towards the end of the third week, then fell again. While this shows that tenuazonic acid is metabolized by the mould, the reason for the double-production peak is obscure.

We are indebted to Imperial Chemical Industries Ltd. for a grant for the purchase of radioactive chemicals.

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Identification of Ohta's Acid ('Tetrahydroxynorsterocholanic acid'). By I. G. ANDERSON and G. A. D. HASLEWOOD (*Guy's Hospital Medical School, London, S.E.* 1)

It has already been suggested that Ohta's acid, originally supposed (Ohta, 1939) to be a 3:6:12:24-tetrahydroxycoprostanic acid, $C_{27}H_{46}O_6$, is in fact a carbon-skeleton isomer of cholic acid (Anderson, Haslewood & Wootton, 1957). A review of the evidence led us to suppose that it might be *allo* (5α)cholic acid.

Methyl Δ^5-3α:12α-diacetoxy-7-oxocholenate was hydrogenated, and, after hydrolysis, a crystalline mixture of acids was obtained. Ethylation gave crystals very similar in properties to mixtures, from natural sources, of ethyl cholate and the ethyl ester of Ohta's acid. This mixture was treated with ethyl 3α:7α:12α-23-tetrahydroxycholanate, isolated from snake bile. A characteristic molecular complex, m.p. about 205°, was obtained, and this was apparently identical with a complex, from snake and seal bile, previously resolved into the ethyl esters of an acid like Ohta's acid and 3α:7α:12α:23-tetrahydroxycholanic acid. Resolution, by partition chromatography on Celite, of the partially synthetic complex gave a sample having an infrared spectrum (KBr disk) between 800 and 1500 cm.$^{-1}$, substantially identical with that of the ethyl ester of Ohta's acid and different from that of ethyl cholate. A new method of preparation and the properties of the methyl ester of Ohta's acid lead us to conclude that this acid is 3α:7α:12α-trihydroxy*allo*(5α)cholanic acid (*allo*-cholic acid).

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The Separation and Quantitative Determination of Amino Acids by High-voltage Paper Electrophoresis. By GLADYS N. ATFIELD and C. J. O. R. MORRIS. (*Department of Experimental Biochemistry, London Hospital Medical College, London, E.* 1)

A scheme has been developed for the separation of eighteen amino acids commonly occurring in protein hydrolysates. The method employs three electrophoretic separations, which are carried out in a 100 cm., double-cooled plate apparatus, similar to that described by Michl (1953) and Gross (1956). A potential gradient of 100 v/cm. is used, and the cooling plates are maintained at 2 ± 1°. Whatman 3MM paper is used throughout.

The first electrophoresis is carried out in a pyridine acetate buffer, pH 6·1, for 150 min., and enables Lys, Arg, His, Asp, Glu and Cy-SO_3H to be separated completely from each other and from the neutral amino acids.

The second separation in 2·5% (w/v) formic acid–7·8% (w/v) acetic acid solution, pH 1·85, for 270 min., resolves Gly, Ala, Ser, Val, Leu + Ileu, Thr, Pro, Glu, Phe + Asp, methionine sulphone, Tyr (and Try if present).

The third electrophoresis in 2% (w/v) formic acid–20% (w/v) acetic acid, pH 1·81, containing 2 mM-cadmium acetate, for 360 min., resolves the amino acid zones, Leu, Ileu (and allo-Ileu if present), and Phe, Asp, which are not separated in the second system.

Following separation the strips are dried at 80° for 15 min. and the amino acid zones located and estimated by a modification of the cadmium ninhydrin method of Heilmann, Barollier & Watzke (1957). Colour development is carried out at room temperature for 24 hr. in the dark. The bright-red zones are cut out together with suitable blank strips, and the pigment eluted with methanol in a special shaking machine. The optical densities of the solutions are determined at 500 mμ, or 352 mμ for proline. The Beer–Lambert law is followed up to an optical density of about 0·8. Appropriate standards are prepared by pipetting 0·1 ml. portions of amino acid solutions on to buffer-treated paper, drying, and developing the red colour as for the electrophoretic strips.

Recoveries of 10–20 μg. amounts of amino acids from mixtures have been in the region of 100 ± 5%.

The applications and theoretical basis of the method are under consideration.

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Biosynthesis of Ubiquinone in the Rat. By D. E. M. Lawson, E. I. Mercer, J. Glover and R. A. Morton. (*Department of Biochemistry, University of Liverpool, Liverpool* 3)

[2-^{14}C]Mevalonate (Gloor & Wiss, 1959) and [1-^{14}C]acetate (Dialameh & Olson, 1959) can be incorporated into ubiquinone in the rat. This involves mainly the isoprenoid side chain, which is probably synthesized before it is coupled to the quinone nucleus in a manner similar to the formation of vitamin K_2 from 2-methyl-1:4-naphthaquinone (Martius & Esser, 1958). Experiments have been carried out with [2-^{14}C]mevalonate to get a measure of the extent of this process in the rat relative to the formation of sterols.

[2-^{14}C]Acetate was injected intraperitoneally into a group (I) of 3 rats on 3 successive days (10 μc/rat/day) and a comparable amount was given orally to a second group (II) of 3 rats over 2 days (16 μc/rat/day). The animals were killed 24 hr. after the last dose, and their livers, intestines and skin removed. The pooled livers, etc., of each group were saponified and known amounts of ubiquinone carrier added. The ^{14}C-labelled ubiquinone, -cholesterol and -lathosterol were resolved chromatographically and finally isolated in pure crystalline form. The specific activities of the ^{14}C-labelled ubiquinone, -mixed sterols, -cholesterol and -lathosterol, were determined. The values for [^{14}C]ubiquinone in skin, intestine and liver of group I were 1150, 1075 and 485 counts/min./mg., respectively, and for group II 800, 30 420 and 2000 counts/min./mg., respectively. This variation between the different tissues suggests that each tissue and probably each cell can incorporate labelled acetate into the ubiquinone. Degradation of the side chain of the pooled ubiquinone fractions by ozonolysis, and isolation of the laevulinic acid as the 2:4-dinitrophenylhydrazone, confirmed that it carried the ^{14}C-label.

Again, on incubating liver slices and everted intestinal sacs in media containing [2-^{14}C]acetate (2·5 μc) and [2-^{14}C]mevalonate (10 μc), respectively, for 3–9 hr., the ubiquinone and sterols became labelled.

In all experiments, the specific activities of the ^{14}C-labelled ubiquinone and -cholesterol were of the same order, but the values for [^{14}C]lathosterol were invariably much higher (3–5:1 for the *in vivo* experiments) and (20–60:1 for the shorter-term *in vitro* experiments). Since the sterol pool is much larger than that of ubiquinone, the turnover rate of the latter must be smaller. The limitation in the process may be the supply of the quinone nucleus rather than the formation of a C_{45}-isoprenoid unit versus the C_{15}-unit used in sterol biosynthesis.

Reversed-phase paper chromatography (Linn *et al.* 1959) of the ubiquinone from rat tissues showed it to contain mainly a C_{45}-isoprenoid side chain, with traces of C_{50}-, C_{40}- and C_{35}-compounds, confirming the finding of Gloor & Wiss (personal communication).

We acknowledge with thanks a gift of [2-^{14}C]mevalonate from Messrs F. Hoffmann-La Roche and Co. Ltd. D. E. M. Lawson is in receipt of a D.S.I.R. studentship, and E. I. Mercer an A.R.C. studentship.

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An Unsaturated Alcohol from Human Kidney. By F. W. Hemming, R. A. Morton and J. F. Pennock. (*Department of Biochemistry, University of Liverpool, Liverpool* 3)

The isolation from human kidney of ubichromenol, a cyclic isomeride of ubiquinone-50, has been described recently (Laidman, Morton, Paterson & Pennock, 1959). From 46·5 kg. of human kidney were obtained 140·7 g. of unsaponifiable material, which on chromatography on Brockmann grade 3 alumina gave a number of fractions. Some of these fractions were found to contain, along with transparent substances, ubichromenol (0·44 g.), ubiquinone-50 (2·70 g.) α-tocopherol (0·22 g.) and β-tocopherol (8 mg.). This paper concerns a compound which was spread over a number of chromatographic fractions, but was intermediate in polarity between ubiquinone and ubichromenol. Its properties have not hitherto been described.

The material (> 5 g.) was a colourless oil at room temperature but it could be crystallized from ethanol at $-30°$. It showed no selective absorption in the ultraviolet region and infrared studies revealed the presence of a hydroxyl group, unconjugated double bonds, and bands indicative of the all-*trans* configuration of these double bonds. A comparison of the infrared-absorption spectrum with that of solanesol (Rowland, Latimer & Giles, 1956) showed many similarities, but some differences were also noted. The human-kidney material

had C, 86·07; H, 12·18%; the iodine number was near 380. Solanesol, $C_{43}H_{73}OH$ (Erickson, Shunk, Trenner, Arison & Folkers, 1959; Kofler *et al.* 1959), requires C, 85·67; H, 11·82%; iodine number 363 (Found: 361).

Thus the unknown material appears to be a long-chain alcohol with similarities to solanesol. It readily forms an acetate which can again be crystallized from ethanol at −30° and other derivatives are now being studied.

This alcohol may have some metabolic importance with regard to ubiquinone and ubichromenol. It is also of interest that Channon, Devine & Loach (1934) and Dimter (1941) isolated a hydrocarbon from pig liver which they suggested was $C_{45}H_{76}$ or $C_{50}H_{84}$. A possible relationship may occur between this and the unknown alcohol.

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Thyroid 'Cysteinyltyrosinase'. By R. E. Loughlin and V. M. Trikojus. (*Department of Biochemistry, University of Melbourne, Australia*)

Extracts of pig-thyroid glands contain an 'acid' proteinase and peptidases (including a carboxypeptidase) which can be partly purified by an acid precipitation procedure followed by acetone fractionation (Laver & Trikojus, 1955, 1956). The proteinase fraction also split L-cysteinyl-L-tyrosine; peptides without this grouping were unaffected. There was also evidence that the two activities are associated with different enzymes, unlike pepsin, which also hydrolyses cysteinyltyrosine (Harington & Pitt-Rivers, 1944). 'Cysteinyltyrosinase' was subsequently separated from proteinase but in low yield, by column electrophoresis (M. T. McQuillan, unpublished experiments).

In further purification studies on cysteinyltyrosinase the acid precipitation step (see above) led to considerable losses in the precipitate (of cysteinyltyrosinase, not proteinase). Furthermore, the enzyme, in extracts of pig thyroids, tended to lose activity on standing, even at 0°, or on dialysis; the addition of thioglycollate had a stabilizing effect.

Bovine thyroids proved to be not only a better source of the enzyme, but the extracts were also more stable to fractionation procedures and dialysis. A 50-fold purification, with about 30% recovery and activity of 31 units per mg. of protein, was achieved as follows. The 35–60% (v/v) acetone fraction from saline extracts was dialysed, adjusted to pH 5·1 and made 0·9% in sodium chloride and the acetone fraction (35–80%) redissolved and dialysed. This solution, adjusted to 35% acetone and 0·9% sodium chloride, was precipitated with zinc acetate, the precipitate dialysed and the supernatant retained. Such preparations are still contaminated with proteinase and work on their further purification is in progress.

The assay of cysteinyltyrosinase is carried out at pH 5·0 in 0·1M-sodium acetate, one unit being defined as the amount which, when incubated with an equal volume of 40 mM-cysteinyltyrosine for 20 min. gives rise to an increase in the extinction at 570 mμ of 1·0 after reaction with ninhydrin. The enzyme also readily splits (CySH).Phe., (CySH).-Tyr.Ileu., Leu.(CySH)., Leu.(CySH).Tyr.NH_2, [on both sides of (CySH)] and Gly.Leu.(CySH).-Tyr.NH_2. Tyr.(CySH). and $(CyS)_2$.$(Tyr.)_2$ are also hydrolysed but more slowly. The preparation of some of these substrates has been reported (Bachelard & Trikojus, 1958).

The enzyme loses activity completely after treatment with 2·25 mM-ethylenediaminetetraacetic acid, followed by dialysis, but may be reactivated by the addition of mM-Zn^{2+}.

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Metabolic Inhibitors and Membrane Permeability. By J. A. HICKLIN (introduced by H. WEATHERALL). (*Department of Pharmacology, London Hospital Medical College, London, E.* 1)

2:4-Dinitrophenol and salicylates uncouple oxidative phosphorylation, (Loomis & Lipmann, 1948; Brody, 1956) and cause a loss of potassium from rat diaphragm (Manchester, Randle & Smith, 1958). It has been inferred that the loss of potassium is due to interference with the active mechanisms responsible for transporting potassium into the cell against the concentration gradient, rather than with passive outward diffusion. Separate analysis of influx and efflux of potassium in rat diaphragm has been made using ^{42}K, and it appears that while 5 mM-sodium salicylate causes a loss of total potassium (final content of the control, 283 mg./kg. dry weight; treated diaphragm, 224 mg./kg. dry weight), the effect is mainly due to an accelerated efflux. In the control the rate constant was 0·0178 min.$^{-1}$; in the treated diaphragm the rate constant was 0·0240 min.$^{-1}$ When allowance is made for this there is no significant difference between the rate of influx of control and treated diaphragms (control influx, 15·5 $\mu\mu$moles/cm.^{2}/sec.; treated-diaphragm influx, 16·3 $\mu\mu$moles/cm.^{2}/sec.). A similar analysis under the influence of 0·5 mM-2:4-dinitrophenol showed the amount of potassium lost to be greater (final content of the control, 247 mg./kg.; in the treated diaphragm, 173 mg./kg.) than with salicylate and there is a much greater acceleration of efflux (control rate constant, 0·0213 min.$^{-1}$; treated-diaphragm rate constant, 0·0498 min.$^{-1}$). Moreover, even when allowance is made in influx experiments for the accelerated rate of efflux in dinitrophenol-treated tissues there is also a considerable slowing of influx (control influx, 16·8 $\mu\mu$moles/cm.^{2}/sec.; treated-diaphragm influx, 10·9 $\mu\mu$moles/cm.^{2}/sec.). The estimations of efflux are more reliable than those of influx, since tissue counts are not subject to error from a surrounding radioactive medium and the rate constant is uninfluenced by movements of tracer in the opposite direction to that under investigation. It is evident that both drugs accelerate efflux, and probable that 2:4-dinitrophenol slows influx, but the latter effect is less notable. It is possible that higher concentrations of salicylate would reduce influx, but those used here (5 mM) are sufficient almost completely to uncouple oxidative phosphorylation (Brody, 1956; Segal & Blair, 1959; Jeffrey & Smith, 1959). As the potassium flux predominantly affected by both drugs is the outward movement from high to low concentrations, it appears that they increase membrane permeability in addition to (or possibly because of) their interference with metabolic activity.

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Isolation of Crystalline D-Amino acid Oxidase Free from Iron. By V. MASSEY and G. PALMER. (*Department of Biochemistry, University of Sheffield, Sheffield* 10)

Kubo *et al.* (1958) reported the preparation of D-amino acid oxidase as the crystalline intact flavoprotein. The absorption spectrum was not that of a typical flavoprotein; the presence of 10 atoms of ferric iron per molecule of flavin was also reported. On adding substrate both iron and flavin were reduced; it was thus concluded that this enzyme is a metalloflavoprotein with the iron participating in electron transfer. In view of the many experimental uncertainties which support the concept of metalloflavoprotein catalysis (Singer & Massey, 1957) it was decided to investigate further the role of iron in this enzyme.

The enzyme was prepared from pig kidney, essentially by the method of Kubo *et al.* (1958), and in agreement with these workers a brown preparation was obtained which contained iron. Also, reduction of the iron on adding alanine was readily shown by chemical analysis (*o*-phenanthroline reaction). That this reduction of iron is merely due to iron-containing impurities acting as electron acceptor was shown by complete removal of the iron without loss of activity. Enzyme prepared by the method of Kubo *et al.* (1958) was adsorbed on a calcium phosphate gel–cellulose column [for details of column method see Massey (1960)] and eluted with 0·05M-phosphate, pH 6·3. The D-amino acid oxidase was eluted as a yellow band, leaving large amounts of reddish-brown iron-containing impurities, still firmly adsorbed at the top of the column. The enzyme was concentrated by ammonium sulphate fractionation and crystallized as

fine needles from ammonium sulphate solution. The visible spectrum of the crystalline enzyme was very similar to that of flavinadenine dinucleotide, except that the absorption peaks were shifted slightly (378 mμ, 462 mμ and a marked shoulder at 485 mμ). The enzyme was completely devoid of iron and under the standard assay conditions of Burton (1955) had an activity of 280 μl. of O_2/min./mg. Adding alanine caused a complete decolorization of the enzyme, after the transient appearance of a red intermediate, presumably the flavin semiquinone (Beinert, 1956). The bearing of these findings on the role of metals in metalloflavoproteins in general and of recent results with electron spin resonance studies (Bray, Malmström & Vänngård, 1959; Beinert & Sands, 1959) was discussed.

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Activation of Trypsinogen by *Penicillium* Kinase. By T. Hofmann. (*Department of Biochemistry, University of Sheffield, Sheffield* 10)

Kunitz (1938) reported that a mould of the genus *Penicillium* produced an enzyme capable of converting trypsinogen into trypsin. Two strains of *Penicillium janthinellum* (IMI 75588 and 75589) which have this property have now been found.

The organisms were grown as surface cultures on the synthetic medium described by Kunitz (1938), with added trace metals. In agreement with Kunitz, it was found that the enzyme appears in the culture medium only after the onset of sporulation.

The enzyme produced by strain IMI 75589 was used exclusively for the following studies. A 20-fold purification was obtained by precipitating the enzyme from the growth medium with ammonium sulphate and dialysing.

The optimum pH of action and the stability of the enzyme suggest that the enzyme is very similar to, if not identical with, Kunitz's enzyme.

In citrate buffer at pH 3·4 it converts trypsinogen quantitatively into trypsin, as shown by the specific activity of the liberated trypsin towards benzoylarginine ethyl ester and towards casein. The trypsin formed is almost completely stable towards *Penicillium* kinase, even on prolonged incubation.

During the activation a number of peptides are produced. They were isolated from the activation mixture by adsorption on 'Amberlite' IR-120 resin and separated after elution by high-voltage electrophoresis. They were identified by paper chromatography of their constituent amino acids and by end-group analysis with fluorodinitrobenzene. In some cases the whole activation mixture was subjected to high-voltage electrophoresis. The following peptides were found:

Val.$(Asp)_4$.Lys, Val.$(Asp)_2$, $(Asp)_2$.Lys, Asp.Asp and free lysine. The hexapeptide Val.$(Asp)_4$.Lys is the same as that found by Davie & Neurath (1955) during autocatalytic activation of trypsinogen. On prolonged incubation the amount of the hexapeptide slowly diminishes. This indicates that at least part of the two tripeptides Val.$(Asp)_2$ and $(Asp)_2$.Lys arises from hydrolysis of the hexapeptide. Asp.Asp and free lysine are only produced in small amounts after prolonged incubation and presumably arise from hydrolysis of $(Asp)_2$.Lys. No free aspartic acid was detected.

Although *Penicillium* kinase is capable of splitting a peptide bond involving the carboxyl group of lysine, it has no measurable activity towards benzoylarginine amide or benzoylarginine ethyl ester. It does not act on acetyltyrosine ethyl ester, but hydrolyses the A-chain of performic acid-oxidized insulin into a number of peptides.

The following compounds (mM) had no effect on trypsinogen activation by *Penicillium* kinase: Cu^{2+}, F^-, ethylenediaminetetra-acetic acid, *p*-chloromercuribenzenesulphonic acid, iodoacetic acid, thioglycollic acid and di*iso*propyl phosphofluoridate.

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The Formation of Phenolic Glycosides by Germinating Broad-bean (*Vicia faba*) Seeds. By J. B. Pridham and M. J. Saltmarsh. (*Chemistry Department, Royal Holloway College, University of London, Englefield Green, Surrey*)

The majority of the naturally occurring low-molecular-weight phenols are present in plant tissues as glycosides (Baruah & Swain, 1957). D-Glucosides are most common although other monosaccharides and short oligosaccharide chains are also found as phenolic derivatives in plants. Previous studies on the biosynthesis of glucosides have been reviewed by Pridham (1960). Hutchinson, Roy & Towers (1958) and Pridham (1958), for example, have infiltrated plant tissues with various phenols and demonstrated the formation of the corresponding glucosides.

It is now shown that glucosides are formed when broad-bean seeds are allowed to germinate between layers of cotton wool which have been soaked in aqueous solutions (1 %, w/v) of various di- and trihydric phenols. Catechol, resorcinol, quinol and phloroglucinol give the corresponding mono-β-glucosides and 2:3- and 2:6-dihydroxyphenyl-β-glucosides are produced on infiltration with pyrogallol. 2-Hydroxybenzyl-β-glucoside is the main product when saligenin (2-hydroxybenzyl alcohol) is used as the substrate.

Cardini & Leloir (1957) found that phenolic glucosides can be produced when uridine diphosphate glucose is incubated with phenols in presence of a wheat-germ extract. This work has been substantiated using enzyme preparations from the bean. Phenolic glucosides apparently cannot be synthesized by transferase reactions involving a phenolic acceptor molecule, a potential glucose donor (e.g. salicin or cellobiose) and β-glucosidase.

The variety and complexity of the naturally occurring plant glycosides suggests that these compounds are biochemically important. The possible function of these compounds *in vivo* is under consideration.

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The Occurrence and Adaptive Increase of 5-Hydroxytryptophan–α-Oxoglutarate Transaminase in Rat Liver. By M. Sandler, R. G. Spector, C. R. J. Ruthven and A. N. Davison. (*Chemical Pathology Department, Queen Charlotte's Maternity Hospital, London, W. 6, and Pathology Department, Guy's Hospital Medical School, London, S.E. 1*)

Blaschko & Hope (1955) suggested that the conversion of 5-hydroxytryptophan into 5-hydroxyindolylpyruvic acid with subsequent oxidation to 5-hydroxyindolylacetic acid might be a possible alternative to the well-established metabolic pathway via 5-hydroxytryptamine. The following evidence tends to support this hypothesis.

Lin, Pitt, Civen & Knox (1958) recently demonstrated a tryptophan transaminase in rat-liver preparations. Their method is based on the principle that enolic tautomers of aromatic α-keto acids form borate complexes, with subsequent shift in keto–enol equilibrium in favour of the enol tautomer. This complex absorbs light more strongly than the same aromatic α-keto acid in the presence of arsenate. The resulting keto–enol difference in optical density is an index of α-keto acid production. Using this technique with minor modifications, we have confirmed their findings in rat liver and have also obtained suggestive evidence of 5-hydroxytryptophan transaminase activity.

When enzyme preparation was incubated with L-tryptophan, α-oxoglutarate and pyridoxal phosphate and portions of the reaction mixture then added to borate and arsenate buffers, respectively, a keto–enol difference absorption spectrum was produced, with maximum optical density at 332 mμ. The curve was identical with that obtained using synthetic indolylpyruvic acid. Substituting DL-5-hydroxytryptophan as substrate, a graph with similar characteristics was obtained, but with a maximum optical density at 340 mμ. This was taken as presumptive evidence of 5-hydroxytryptophan transaminase activity. It was not possible to confirm the presence of 5-hydroxyindolylpyruvic acid by comparing the curve with that of an authentic sample, for this compound, which is likely to be unstable, has not to our knowledge been produced synthetically.

Although a higher activity was observed using tryptophan as substrate, the possible identity of the two enzymes is suggested by the following data. (1) An adaptive increase in tryptophan transaminase following injection of its substrate has been described by Civen & Knox (1959). Such adaptive increase in concentration of both enzymes was produced by injection of either mM-L-tryptophan or 2 mM-DL-5-hydroxytryptophan, 5 hr. prior to killing the rat. Lin & Knox (1958) have pointed out, however, that adaptive enzyme formation is not specific to its substrate. (2) The optimum pH for both reactions was 8·2. (3) The activity of both enzymes, which are highly thermolabile, diminished rapidly and to a similar degree at room temperature.

We are grateful to Professor H. Veldstra for a gift of indolylpyruvic acid.

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Does Dietary Galactose Play a Part in the Synthesis of Brain Lipids of the New-born? By S. N. Varma and V. Schwarz. (*Departments of Pathology and Child Health, University of Manchester, Manchester* 13)

The rapid development of the new-born mammal's brain, including the synthesis of galactolipids, normally coincides with the ingestion of relatively large amounts of galactose. This has prompted the suggestion that dietary galactose plays a definite role in the synthesis of galactose-containing compounds of the nervous system (Platt, 1955). Several authors have speculated on the apparent correlation of the lactose content of the milk and the cerebroside content of the brain or the degree of cerebral development in different species (Sadhu, 1948, 1952; Ziegler, 1956).

Studies, *in vivo* and *in vitro*, have indicated the potential ability of the brain to incorporate both glucose and galactose into galactolipids (Radin, Martin & Brown, 1957, Burton, Sodd & Brady, 1958). The following study was designed to establish whether normal brain development is possible, despite the virtual deprivation of galactose, such as occurs in hereditary galactosaemia (Kalckar, 1957; Holzel, Komrower & Schwarz, 1957) and possibly 'Alactasia' (Holzel, Schwarz & Sutcliffe, 1959).

Kittens were hand-fed from birth to various ages up to the normal weaning time. Thirty-one animals, from nine litters, were fed one of two diets containing maltose and lactose, respectively, as the sole carbohydrate, The animals grew and developed normally, the weight gains being similar to those of control animals left with their mothers. The brains were in all cases normal in weight and histological appearance. The following determinations were made: total brain galactose, glucose, choline, neuraminic acid, lipids and solids. There was no significant difference in any of the parameters, with the possible exception of choline, between the two groups of animals.

Human brains derived from three galactosaemic infants and eight 'controls' were similarly investigated.

The relevance of these findings to the synthesis of galactolipids in the brain is under consideration.

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Fatty Acids of Phospholipids from Mitochondria and Microsomes of Rat Liver. By M. G. Macfarlane, G. M. Gray and L. W. Wheeldon. (*Lister Institute of Preventive Medicine, London, S.W.* 1)

Mitochondrial and microsomal fractions were prepared from rat liver homogenized in 0·25 M-sucrose, by successive centrifugation at 600, 1200 and 78 000 *g*. The lipid was extracted with 19 vol. of chloroform–methanol (2:1, v/v) and fractionated on silicic acid by elution with increasing concentrations of methanol in chloroform. The phospholipids of mitochondria and microsomes, which in both constituted about 75% of the lipid, comprised respectively (as % of the lipid): cardiolipin, 9

and 1; inositide, 6 and 10; kephalin, 31 and 26; lecithin, 51 and 62.

The fatty acids of the main kephalin fraction and of three successive equal fractions constituting the lecithin band were analysed by gas chromatography of the methyl esters on 'Reoplex 400'. The fatty acids of the mitochondrial kephalin were (as % of the total): C_{12}, C_{15} and C_{17} acids, 2; palmitic, 18; stearic, 30; palmitoleic, 1; oleic, 5; linoleic, 4; C_{20} trienoic 1; C_{20} tetraenoic (arachidonic), 21; C_{20} pentaenoic, 2; C_{22} pentaenoic, 2; C_{22} hexaenoic, 14. The microsomal kephalin was almost identical.

The fatty acids of the mitochondrial lecithin were (as % of the total): C_{14}, C_{15}, C_{17} and C_{19} acids, 2; palmitic, 13; stearic, 22; palmitoleic, 4; oleic, 14; linoleic, 20; C_{20} trienoic, 2; C_{20} tetraenoic, 14; C_{20} pentaenoic, 2; C_{22} pentaenoic, 1; C_{22} hexaenoic, 8. The successive lecithin fractions all contained about 35% of saturated acids, but the proportion of C_{20} and C_{22} acids decreased, and of oleic and linoleic acids increased, through the band. The microsomal lecithin was almost identical. Linolenic acid was not present in the kephalin or lecithin.

About 45% of the fatty acids of the kephalin and lecithin of mitochondria and microsomes of rat liver are 'essential' fatty acids, reckoned as the sum of linoleic and C_{20} and C_{22} polyenoic acids. Since cardiolipin and inositide are also rich in these acids (Faure, Morelec-Coulon, Maréchal & Leborgne, 1959) all the phospholipids are susceptible to peroxidation (cf. Tappel & Zalkin, 1959; Horgan, Philpot, Porter & Roodyn, 1957). The integrity of the lecithin is essential for the succinoxidase activity of mitochondria (Macfarlane, 1950; Nygaard & Sumner, 1953; Macfarlane & Datta, 1954), so inability to form the necessary lecithin on a diet deficient in essential fatty acids might alone limit expansion of the oxidative and synthetic capacity necessary for growth, but presumably the other phospholipids are also of functional significance.

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DEMONSTRATIONS

Electrophoresis of Plasma Proteins in Starch Gels. By H. Harris, D. G. Penington and E. B. Robson. (*Department of Biochemistry and Medical Unit, London Hospital Medical College, London, E.* 1)

The demonstration is mainly concerned with the genetically determined variants of human haptoglobin, transferrin and serum albumin. New points are:

(1) The presence of a minor component of human transferrin which migrates towards the anode at pH 8·6 more slowly than the major component (transferrin C). The minor component appears to be present in most normal sera but is rather variable in amount. It can account for only a small fraction of the total iron-binding capacity. However, its iron-binding capacity as judged from radioautographs, when compared with that of transferrin C, is roughly in proportion to the relative intensities of staining of these two proteins with naphthalene black.

(2) The occurrence of a new, rare, genetically determined variant of transferrin with mobility intermediate between that of Tf D_0 and Tf D_1 (Giblett, Hickman & Smithies, 1959). This has been found in association with Tf C in several individuals in two different families of European origin. In these individuals the unusual transferrin component was present in amounts about equal to Tf C, which itself occurred in about half the quantity normally present.

(3) The treatment of serum proteins with iodoacetamide under various conditions resulting in characteristic changes in the mobilities of certain components. Serum albumin shows an appreciably lower mobility towards the anode at pH 8·6. This is possibly attributable to blocking of the free sulphydryl groups. Transferrin C may be converted into two distinct slower-moving components. Both of these retain the ability to bind iron, and the total iron-binding capacity is not significantly reduced. The transferrin variants D_1 and D_2 behave in a similar manner. Thus, in the heterozygote Tf CD_2, four new components may be observed. The haptoglobins, prealbumins and slow α^2

components (Smithies, 1955) are not obviously affected by the treatment.

(4) The comparison of the pattern of haptoglobin components in serum and urine from patients with proteinuria. This often reveals peculiarities in haptoglobin pattern in the urine. In the 2-1 phenotype the relative concentrations of the faster-moving (and presumably lower-molecular-weight) components are usually markedly increased compared with the slower-moving (and presumably higher-molecular-weight) components. The urinary haptoglobin pattern often resembles that seen in sera of individuals with the so-called modified 2-1 phenotype (Connell & Smithies, 1959). However, the plasma haptoglobin pattern is generally not obviously distorted. Similar changes may occur in the 2-2 phenotype.

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Photography of Gels from Starch-Gel Electrophoresis. By RUTH JACOBS (introduced by F. L. WARREN). (*Department of Biochemistry, London Hospital Medical College, London, E.* 1)

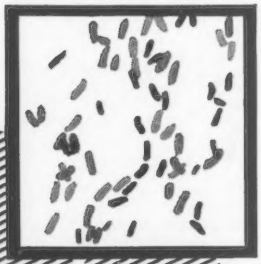
The coloured bands produced by staining proteins separated by starch-gel electrophoresis with dyes such as naphthalene black, nigrosine, or benzidine, fade on storage of the gels. Photographic reproduction of the freshly stained gel allows the permanent recording of fine detail for later study.

As a routine, starch gels are photographed on 35 mm. film (Recordak Micro-file, Kodak Ltd.) with a Shackman auto-camera. The gels are illuminated by two 150w. lamps placed about 40 cm. from the centre of the gel and at an angle of about 45°. The gels are photographed while immersed in their final washing fluid.

Under these conditions good negatives are obtained with the following exposures: naphthalene black gels, 8 sec. at *f*8 with red (×8) filter; nigrosine gels, 3 sec. at *f*11 with green (×3) filter; benzidine gels, 5 sec. at *f*8 with red (×8) filter.

With negatives made in this way prints on hard grades of bromide paper show good definition, even of bands which appear weak in the original.

Erwinia Chrysanthemi:
A plant pathogen causing Stem Rot in Chrysanthemums and Soft Rot in fleshy vegetables



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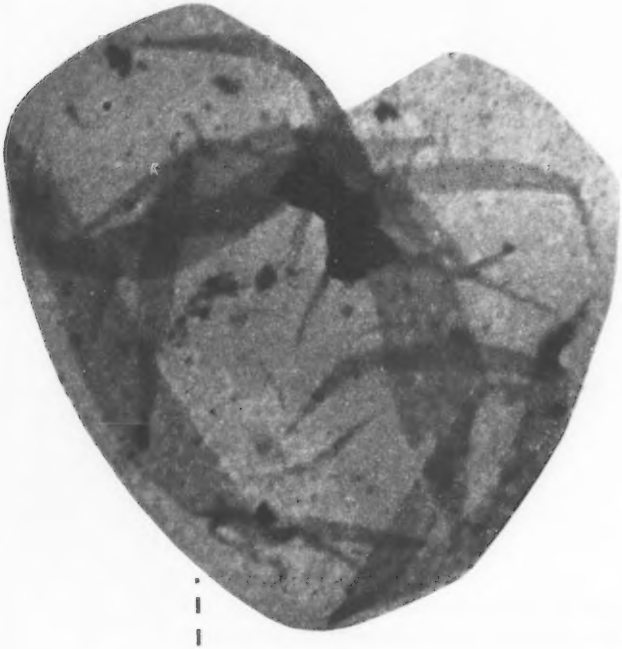

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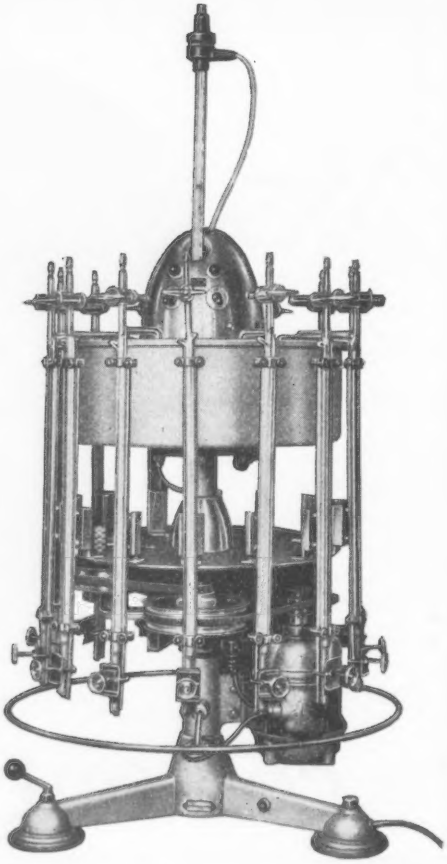

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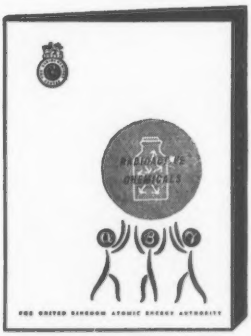

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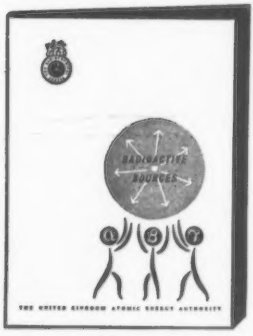

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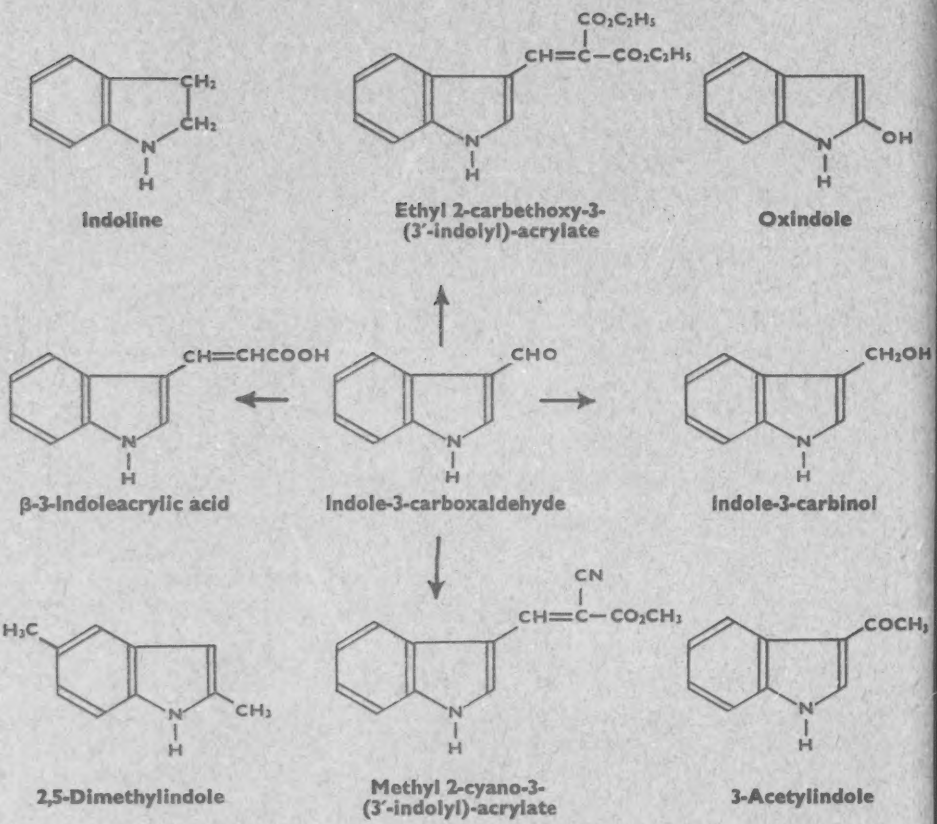

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